

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 48/00, C12N 15/00, C07K 14/00, C07H 21/00		A1	(11) International Publication Number: WO 95/09011
			(43) International Publication Date: 6 April 1995 (06.04.95)
(21) International Application Number: PCT/US94/11111			(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).
(22) International Filing Date: 30 September 1994 (30.09.94)			
(30) Priority Data: 129,391 30 September 1993 (30.09.93) US			
(71) Applicant: UNIVERSITY OF PENNSYLVANIA [US/US]; Center for Technology Transfer, Suite 300, 3700 Market Street, Philadelphia, PA 19014-3147 (US).			
(72) Inventors: SCHREIBER, Alan, D.; 821 Westview Street, Philadelphia, PA 19119 (US). PARK, Jong-Gu; 6813 Drexelbrook Drive, Drexel Hill, PA 19026 (US).			
(74) Agent: WILSON, Mary, J.; Nixon & Vanderhye, 8th floor, 1100 North Glebe Road, Arlington, VA 22201-4714 (US).			Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHODS OF STIMULATING PHAGOCYTOSIS			
(57) Abstract <p>The present invention relates, in general, to methods of stimulating phagocytosis and thereby combating infection and/or modulating immune complex disease, in particular, to methods of modulating the number and type of Fc receptors present on cells that normally possess such receptors, including monocytes and macrophages, as well as on cells that normally do not possess Fc receptors, such as fibroblasts, and to compounds and compositions suitable for use in such methods.</p>			

BEST AVAILABLE COPY

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

METHODS OF STIMULATING PHAGOCYTOSIS

This is a continuation-in-part of Application No. 08/129,391, filed September 30, 1993, the contents of which are incorporated herein by reference.

5

TECHNICAL FIELD

The present invention relates, in general, to methods of stimulating phagocytosis and thereby combating infection and/or modulating immune complex disease, in particular, to methods of modulating the number and type of Fc receptors present on cells that normally possess such receptors, including monocytes and macrophages, as well as on cells that normally do not possess Fc receptors, such as fibroblasts, and to compounds and compositions suitable for use in such methods.

15

BACKGROUND

Mononuclear phagocytes (blood monocytes and tissue macrophages) have cell surface receptors for the Fc domain of IgG antibody. These receptors (FcγR) mediate humoral immune effector functions including phagocytosis, clearance of immune complexes and antibody-dependent cell cytotoxicity. Three classes of Fcγ receptors have been identified on human cells and characterized on the basis of size, primary structure, binding affinity for IgG subclasses, and recognition by

20

25

monoclonal antibodies: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRI is a high affinity receptor, expressed on resting mononuclear phagocytes and stimulated neutrophils. FcγRII and FcγRIII are low affinity receptors found on a range of hematopoietic cells, including monocytes and macrophages. Macrophages express all three receptor classes while monocytes express primarily FcγRI and FcγRII.

All three classes of human Fcγ receptors have been isolated and cloned (Allen and Seed, Science 243:378 (1989); Hibbs et al, Proc. Natl. Acad. Sci. USA 85:2240 (1988); and J. Exp. Med. 166:1668 (1987)). At least two genes code for the FcγRI class of receptors (van de Winkle et al, FASEB J. 5:A964 (1991)), three genes code for the FcγRII class (designated FcγRIIA, FcγRIIB and FcγRIIC) (Brooks et al, J. Exp. Med. 170:369 (1989); Stuart et al, EMBO J. 8:3657 (1989); Qui et al, Science 248:732 (1990)) and two genes code for the FcγRIII receptor class (Simmons and Seed, Nature 333:568 (1988)).

Macrophage Fcγ receptors participate in the clearance of IgG-coated particulate and soluble antigens, including IgG-coated microorganisms, and thereby remove potentially dangerous foreign organisms. Due to their importance in host defense, functional integrity of Fcγ receptors has been studied in connection with various disease states, including autoimmune disorders (Frank et al, Ann. Intern. Med. 98:206 (1983); Kimberley and Ralph, Am. J. Med. 74:481 (1983)) and end-stage renal disease (Ruiz et al, N.

Engl. J. Med. 322:717 (1990)). Macrophage Fc γ receptor function has been found to be decreased in patients with certain HLA haplotypes and in patients with the immune disorders systemic lupus erythematosus, Sjogren's syndrome and dermatitis herpetiformis (this observation was attributed to occupation of these receptors on the macrophages by immune complexes). In end-stage renal disease, macrophage Fc γ receptor function has been found to be impaired and this impairment is believed to contribute to the observed immunodepression among such patients.

Various diseases, non-bacterial in origin, are associated with a high incidence of complications due to infection. Examples of such diseases include the above-noted end-stage renal disease (Goldblum and Reed, Ann. Intern. Med. 93:597 (1980); Lahnborg et al, Transplantation 28:111 (1979); Drivas et al, Invest. Urol. 17:241 (1979); Keane and Raij, In: Drukar et al eds. Replacement of Renal Function by Dialysis, 2nd ed., pp. 646-58 (1983)), acquired immunodeficiency syndrome (AIDS) (Bender et al, J. Infect. Disease 152:409 (1985), Smith et al, J. Clin. Invest. 74:2121 (1984)), liver disease (Rimola, In: McIntyre et al eds Oxford Textbook of Clinical Hepatology, pp. 1272-84 (1991)) and diseases of the lung, including cystic fibrosis (Gomez and Schreiber, unpublished observations) and acute respiratory distress syndrome (ARDS) (Rossman et al, Clin. Res. 41:251A (1993)). Defective Fc γ receptor-dependent clearance has been observed in certain of these diseases. Thus, there is

a clear need for methods that can be used to correct defective Fc γ receptor function and/or enhance functional Fc receptor expression and thereby stimulate host defense. The present invention provides such methods and compounds and compositions suitable for use therein.

OBJECTS AND SUMMARY OF THE INVENTION

It is a general object of the present invention to provide a method of combating infection by stimulating phagocytosis.

It is the specific object of the invention to provide a method of stimulating phagocytosis by modulating the number and type of Fc receptors present on cells that normally possess such receptors, including monocytes and macrophages. In addition, it is a specific object of the invention to provide a method of combating infection by rendering cells phagocytic that do not normally possess that function, such as fibroblasts or epithelial or endothelial cells not normally expressing Fc γ receptors.

It is a further object of the invention to provide constructs suitable for use in gene therapy protocols that encode Fc receptors, and cells transformed therewith.

In one embodiment, the present invention relates to a method of increasing the phagocytic potential of cells present in a mammal that comprises introducing into the cells a DNA molecule coding for an Fc

receptor. The introduction is effected under conditions such that the DNA molecule is expressed, the Fc receptor produced, and the phagocytic potential of the cells thereby increased.

5 In a further embodiment, the present invention relates to a method of increasing the phagocytic potential of cells of a mammal that comprises:

- i) removing cells from the mammal,
- ii) introducing into the cells a DNA molecule
10 encoding an Fc receptor, and
- iii) reintroducing the cells into the mammal under conditions such that the DNA molecule is expressed, the Fc receptor produced, and the phagocytic potential of the cells thereby increased. One skilled
15 in the art will appreciate that steps (i)-(iii) can be carried out using methodologies known in the art.

In other embodiments, the present invention relates to a liposome comprising a DNA molecule encoding an Fc receptor, a bacterium comprising a DNA
20 molecule encoding an Fc receptor, a T cell comprising an exogenous DNA sequence encoding an Fc receptor, and a B cell comprising an exogenous DNA sequence encoding an Fc receptor.

In yet another embodiment, the present invention
25 relates to a DNA construct encoding an Fc receptor comprising domains, or functional portions thereof, from at least two of FcγRI, FcγRII and FcγRIII, wherein the domains, or portions thereof, are such that the receptor renders cells phagocytic that comprise same.
30 The invention also relates to the encoded Fc receptor.

In a further embodiment, the present invention relates to a method of treating an infection comprising administering to a mammal in need of such treatment a DNA molecule encoding an Fc receptor. The administration is effected under conditions such that the DNA molecule is expressed in cells of the mammal, the Fc receptor produced, and the phagocytic potential of the cells thereby increased. The resulting cells phagocytose IgG-coated particles causing the infection, or IgG-containing soluble immune complexes derived from the infection.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A and 1B - A) Biotinylation of D58 (Src+) and SAR6 (Src-) cells infected with FcγRIIA. Immunoprecipitation with anti-FcγRII mAb IV.3 demonstrates the 40 kD FcγRIIA protein in the membrane of FcγRIIA-infected cells (lanes 2 and 4). No receptor is present in the sham-infected cells (lanes 1 and 3). B) Phosphorylation of FcγRIIA on tyrosine after receptor crosslinking in FcγRIIA-infected D58 and SAR6 cells. Phosphotyrosine containing proteins were immunoprecipitated from cell lysates with and without FcγRIIA stimulating (+EA and -EA). Induction of the tyrosine phosphorylated 40 KD receptor is seen in lanes 6 and 8.

FIGURES 2A and 2B - Fluorescence histograms of (A) D58 and (B) SAR6 cells infected with FcγRIIA. The dotted line represents cells stained with an isotype control mAb and the solid lines represent cells stained with anti-FcγRII.

FIGURES 3A and 3B - *In vitro* immune complex kinase assay of Src related tyrosine kinases from FcγRIIA infected D58 (Src+) (lanes 1-6) and SAR6 (Src-) cells (lanes 7-12). FcγRIIA-infected and sham-infected cells were lysed and cell lysates immunoprecipitated with the antibodies indicated above each lane (RAM is the rabbit-anti-mouse control, IV.3 is anti-FcγRII mAb, Src and Fyn are mAbs specific for these kinases). Immune complexes were exposed to [γ^{32} P]ATP to allow autophosphorylation of the kinases and phosphorylation of FcγRIIA. The positions of the phosphorylated Src, Fyn and FcγRIIA proteins are indicated by the open squares, stars and arrows, respectively. Lanes 2 and 8, representing immunoprecipitates with Src antibody alone, confirm the Src+ and Src- phenotypes of the D58 and SAR6 cell lines.

FIGURE 4 - Macrophage Fcγ-receptor-mediated clearance of IgG-sensitized radiolabeled red cells in patients with alcoholic cirrhosis of the liver (n=49), non-cirrhotic alcoholic subjects (n=10) and healthy volunteers. The middle three curves (means \pm SEM) represent values for clearance of IgG-sensitized red cells in these 79 subjects; the upper pair of curves,

the clearance of unsensitized autologous red cells in five patients and five controls; and the lower pair of curves, the clearance of heat-damaged red cells (heated for 30 minutes at 56°C) in five patients and five controls.

FIGURE 5 - Macrophage Fcγ-receptor-mediated clearance of IgG-sensitized radiolabeled red cells in patients with alcoholic cirrhosis of the liver (n=49), and healthy volunteers (n=20). The four middle curves (means ± SEM) represent values for clearance in these 69 subjects: patients with mildly decompensated alcoholic cirrhosis of the liver (cirrhosis I, n=17), patients with moderately decompensated alcoholic cirrhosis of the liver (cirrhosis II, n=17), patients with severely decompensated alcoholic cirrhosis of the liver (patients III, n=15), and controls (n=20).

FIGURE 6 - Macrophage Fcγ-receptor-mediated clearance of IgG-coated red cells (as half-time) in patients with alcoholic cirrhosis of the liver (n=49) and in controls (n=20). The half-time was significantly longer in the eleven patients in whom severe infection developed during follow-up.

FIGURE 7 - Recognition of human IgG(anti-RhD)-coated red cells by monocytes from patients (n=49) and controls (n=20). IgG-sensitized, ⁵¹Cr-labeled (2×10^7) erythrocytes were added to monolayers of monocytes, and the percentage of red cells bound by monocytes was

determined by measuring the radioactivity. Values are means \pm SEM.

FIGURE 8 - Recognition of mouse IgG2b-coated red cells by monocytes from patients (n=49) and controls (n=20). IgG2b-sensitized erythrocytes were added to monolayers of monocytes, and the percentage of monocytes binding >3 RBC per cell was determined. Values are means \pm SEM.

FIGURE 9 - Macrophage Fc γ -receptor-mediated clearance in patients with circulating immune complexes (n=7). The curves for these patients fell into the range for the patient group.

FIGURE 10 - Tyrosine phosphorylation in wild type J32 and in mutant J32-3.2 transfectants.

Antiphosphotyrosine immunoblots were prepared following immunoprecipitation of cell lysates with either anti-phosphotyrosine antibody or anti-Fc γ RII antibody. The 40 kD Fc γ RII receptor is phosphorylated on tyrosine following Fc γ RII activation.

FIGURES 11A and 11B - Fluorescence histograms of J32/Fc γ RIIA and J32-3.2/Fc γ RIIA stable transfectants, and Fc γ RIIA expressing clones. Flow cytometry was employed with anti-Fc γ RII monoclonal antibody IV.3 or with an isotype control (Indik et al, J. Clin. Invest. 88:1766 (1991)).

FIGURES 12A and 12B - Phagocytosis of IgG coated erythrocytes by J32 and J32-3.2 transfectants. EA was prepared as described previously (Indik et al, J. Clin. Invest. 88:1766 (1991)), overlaid onto transfected or sham-transfected T-cells and incubated at 37°C for 30 minutes. Unbound EA was removed by washing with PBS and extracellular bound EA was removed by exposure to hypotonic buffer before staining with Wright-Geimsa.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of modulating the phagocytic potential of cells that are naturally phagocytic, such as macrophages, and to methods of rendering cells phagocytic that do not naturally possess that function. In so doing, the present invention provides innovative treatment regimens that can be used to combat infections associated with various disease states.

Drug Induced Enhancement of Fcγ Receptor Expression:

In one embodiment, the present invention relates to a method of enhancing Fcγ receptor expression on phagocytic cells of a mammal, including macrophages. The method comprises administering to the mammal an active agent, such as the cytokine interferon gamma (IFN-γ), an estrogen or estrogen analog, or a hematopoietic growth factor such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or

macrophage colony stimulating factor (M-CSF). IFN- γ has been shown to modulate the levels of Fc γ RI and Fc γ RII apparently by increasing gene transcription. Dexamethasone has been reported to influence this IFN- γ -induced enhancement of transcription in a cell-specific manner (Comber et al, Cell. Immunol. 145:324 (1992)). Estradiol and diethylstilbesterol have been shown to facilitate clearance of IgG-coated cells (Friedman et al, J. Clin. Invest. 75:162 (1985); Ruiz et al, Clin. Res. 38:367A (1990)). GM-CSF has been shown to selectively increase monocyte Fc γ RII expression and function (Rossman et al, Exp. Hematol. 21:177 (1993)), and, similarly, M-CSF has been shown to increase splenic macrophage Fc γ receptors and thereby enhance the clearance of IgG-coated cells (Ruiz et al, Clin. Res. 40:796A (1992)).

One or more of the above-referenced active agents can be combined with an appropriate carrier to form a dosage form suitable for use in the method of the present invention. The amount administered will vary depending on the patient, the agent, the clinical response sought and the route of administration. Appropriate concentrations and dosage regimens can be readily determined by one skilled in the art having knowledge of these agents.

The active agents can be formulated as capsules, tablets, and the like, and as solutions and suspensions suitable for intravenous or parenteral administration. The agents can also be formulated as aerosols for administration to the lung. Carriers used are

pharmaceutically acceptable and depend on the dosage form.

In vivo synthesis of the above active agents can be effected, for example, at a particular site, by introducing into cells of the patient sequences encoding the agent in an appropriate vector (e.g. an adenoviral or retroviral vector) preferably in combination with an Fc γ receptor encoding sequence (see below). In a preferred embodiment, the sequence encoding the agent encodes M-CSF and the sequence encoding the receptor encodes the γ chain of Fc γ RIII. Such encoding sequences can also be administered, for example, in liposomes, particularly where lung is the target tissue.

Conditions amenable to treatment by the above-noted active agents include those characterized by reduced macrophage Fc γ receptor number or function, for example, chronic renal failure, liver disease and pulmonary disorders, including acute respiratory distress syndrome (ARDS), AIDS and cystic fibrosis. Such agents can be used in combination with one or more of the therapeutic approaches described below to enhance Fc γ receptor activity and thereby treat infections that often accompany these conditions and others.

Fc γ Receptor Gene Therapy:

In a further embodiment, the present invention relates to the use of recombinant and gene therapy

protocols to modulate Fc receptor expression. As noted above, genes encoding all three classes of Fcγ receptors have been isolated and cloned. All three receptor classes, FcγRI, FcγRII and FcγRIII, consist of distinct domains corresponding to their location within the cell. The cDNA structure of the FcγRII class of receptors, for example, consists of a 5' untranslated region, sequences coding for a signal peptide region (S), an extracellular domain (EC), a transmembrane region (TM), an intracytoplasmic domain (C), and a 3' untranslated region (Schreiber et al, Clin. Immunol. Immunopath., 62:S66 (1992), Cassel et al, Molec. Immunol. 30:451 (1993)). Likewise, the predicted polypeptide sequence of FcγRI shows a hydrophobic signal sequence, a hydrophobic transmembrane region and a charged cytoplasmic domain, in addition to an extracellular region that consists of three immunoglobulin-like domains, two of which share homology with the other Fcγ receptors (Allen and Seed, Science 243:378 (1989); Schreiber et al, Clin. Immunol. Immunopath., 62:S66 (1992)). FcγRIIIA is a complex consisting of a single α chain and a homo- or heterodimer of associated γ and ζ chains (Letourneur et al, J. Immunol. 147:2652 (1991); Ra et al Nature (Lond.) 241:752 (1989); Park et al, Clin. Res. 41:324A (1993)). Both the γ and ζ chains mediate phagocytosis, the γ chain being more efficient (Park et al, Clin. Res. 41:324A (1993)). The extracellular domain of FcγRIII is closely homologous to that of FcγRI and FcγRII, however, the transmembrane domain of FcγIII terminates

in a 200-220 residue hydrophobic domain followed by four hydrophobic residues, one of which is charged (Simmons and Seed, Nature 333:568-570 (1988)). FcγRIII thus differs from FcγRI and FcγRII in that the latter
5 two have substantial intracellular cytoplasmic domains.

FcγRI is unique among the three classes of human Fcγ receptors not only in its high affinity for IgG but also in the structure of its cytoplasmic domain. Macrophage FcγRII and the γ chain of FcγRIII have
10 tyrosine residues in their cytoplasmic domains that are required for phagocytosis. In contrast, FcγRI does not contain tyrosine residues in its cytoplasmic domain (Allen and Seed, Science 243:378 (1989)) and is not phosphorylated on tyrosine. Further, FcγRI is unusual
15 among the Ig gene family of receptors in not requiring its cytoplasmic domain for phagocytosis (Indik et al, Clin. Res. 41:170A (1993)).

Recombinant techniques make it possible to manipulate the domains of naturally occurring receptors and thereby design Fc receptors having specific
20 characteristics. The present invention contemplates the use in gene therapy regimens of DNA sequences encoding such selectively constructed receptors, comprising domains from single or multiple Fcγ
25 receptors, to effect the production of receptors having defined activities, both in cells that normally produce Fcγ receptors and in cells that normally do not. In the former case, the Fc receptor sequence introduced into target cells can encode a protein essentially
30 identical to that normally produced by the cell.

Alternatively, the sequence introduced can encode: i) an Fc receptor protein that is functionally comparable to, but structurally different from, the naturally occurring receptor (e.g. a protein comprising only functional portions of the domain(s) (for example, the cytoplasmic domain) of the naturally occurring receptor), or ii) a receptor protein that differs functionally and structurally from the Fc receptor that is normally present on the cell (e.g. a chimeric receptor protein comprising a high affinity Fc γ RI extracellular domain and transmembrane and cytoplasmic domains from Fc γ RIIA or Fc γ RIIIA). The present invention thus makes it possible to compensate for deficiencies in the production of Fc receptors of a particular functional type, which deficiencies may occur in association with a particular disease state. The invention also makes it possible to manipulate the composition of the Fc receptor population of a particular cell type. That is, a cell producing predominantly high affinity receptors can be engineered so as to produce predominantly low affinity Fc receptors.

The transmission of extracellular signals to cellular targets by many surface receptors is dependent upon interaction between cytoplasmic protein tyrosine kinase and tyrosine-containing sequences in the cytoplasmic domain of the receptor, or an associated subunit. The *in vivo* kinase important for γ chain mediated phagocytosis is Syk. The data presented in Example XI make it clear that transfection or

cotransfection of a Syk encoding sequence can be used to enhance phagocytosis mediated by the γ chain (as well as by the ξ chain, in a target cell. Various constructs can be used for this purpose.

5 Equally important, the present invention makes it possible to render cells phagocytic that do not normally possess that function. Sequences encoding naturally occurring Fc γ receptors or sequences encoding non-naturally occurring Fc receptors, for example,
10 chimeric receptors that include entire domains, or functional portions thereof, from two or more naturally occurring Fc γ receptors, can be introduced into such cells. The chimeric receptors can be designed so as to take into account both the phagocytic potential of the
15 cells into which the encoding sequences are to be introduced and the receptor domain properties suited for achieving the desired therapeutic effect. While not all cells are equally suitable as recipients for all Fc receptor-encoding constructs, operability can be
20 readily assessed using *in vitro* model systems such as those described by Indik et al (J. Clin. Invest. 88:1766 (1991) and Hunter et al, Clin. Res. 41:244A (1993); see also Amigorena et al, Nature (Lond) 358:337 (1992); Park et al, Clin. Res. 41:324A (1993); Toijman
25 et al, Blood 79:1651 (1992); Kruskal et al, J. Exp. Med. 176:1673 (1992); (see also Examples below)). This embodiment of the invention may be particularly advantageous since cells, such as fibroblasts, that are rendered phagocytic may ingest particles without
30 releasing significant quantities of superoxide radicals

or toxic biologically active products. This is in contrast to cells that are normally phagocytic, such as macrophages. One skilled in the art will appreciate that a reduction in the release of toxic products results in a reduction in the possibility of inflammation.

Constructs:

Chimeric Fc receptors suitable for use in the present invention include those prepared as detailed in the Examples below. For instance, single chain chimeras of the α and γ chains of FcRIIIA can be prepared. Sequences encoding such chimeras have been introduced into COS-1 cells and the phagocytic potential conferred examined. For example, a DNA sequence encoding the extracellular domain of the α chain of FcRIIIA, the transmembrane domain of the γ chain of FcRIIIA or Fc γ RI and the cytoplasmic domain of the γ chain of Fc γ RIIIA has been transfected into COS-1 cells (the transmembrane domain of the α chain of Fc γ RI can be used in lieu of that of the γ chain, though perhaps not as effectively). Such chimeras display phagocytic activity in the COS-1 assay system though not at a level equivalent to the multichain form of FcRIIIA. In spite of the reduced activity, single chain constructs are clearly advantageous in view of the difficulties inherent both in introducing into target cells multiple sequences and in achieving proper complexation of the encoded proteins.

Fc chimeric receptors have also been prepared from a combination of domains of Fc γ RII isoforms and from a

combination of FcγRI and FcγRII domains. Specifically, a chimeric receptor comprising the extracellular and transmembrane domains of FcγRIIB2 and the cytoplasmic domain of FcγRIIA has been shown to confer phagocytic potential on host cells, thus demonstrating that the FcγRIIB2 transmembrane domain is capable of transmitting the phagocytic signal to the FcγRIIA cytoplasmic domain (FcγRIIB receptors do not themselves confer phagocytic potential). Similarly, a chimeric receptor comprising the extracellular domain of FcγRI and the transmembrane and cytoplasmic domains of FcγRIIA has been shown to induce phagocytosis in host cells. In contrast, chimeras comprising the extracellular domain of FcγRI and the transmembrane domain of FcγRI or FcγRIIA do not result in phagocytosis when the cytoplasmic domain is from FcγRIIA or FcγRI, respectively. However, chimeras comprising the extracellular domain of FcγRI, the transmembrane domain of FcγRI and the cytoplasmic domain of the γ chain of FcγRIII, do result in phagocytosis. It will be appreciated that chimeras comprising the extracellular domain of FcγRI (and appropriate transmembrane and cytoplasmic domains) can be advantageous in view of the high binding affinity of the FcγRI extracellular region.

Chimeras in addition to those described above and detailed below are contemplated. For example, the cytoplasmic domain of FcγRIIA can be used in combination with the extracellular domain of FcγRI and the transmembrane domain of FcγRIIA. Further, the

extracellular and transmembrane domains of FcγRI or FcγRII can be used in combination with the cytoplasmic domain of the γ chain of FcγRIII. Further, chimeras of the invention can include the extracellular domain from FcγRIIA, FcγRI or from the α chain of FcγRIII, the transmembrane domain from FcγRIIA or from the α or γ chain of FcγRIII, and the cytoplasmic domain of either the γ chain of FcγRIII or FcγRIIA (e.g., i) the extracellular and transmembrane domains of FcγRIIA, ii) the extracellular domain of the α chain of FcγRIII and the transmembrane domain of the γ chain of FcγRIII, or iii) the extracellular domain of FcγRI and the transmembrane domain of the α or γ chain of FcγRIII - each with the cytoplasmic domain from either the γ chain of FcγRIII or FcγRIIA (it is noted that preliminary results suggest that certain chimeras comprising the transmembrane domain of the α chain of FcγRIII may not be operative).

While chimeras of the invention can include the entire extracellular, transmembrane and cytoplasmic domains of the respective naturally occurring receptors, such is not necessarily the case. Rather, the chimeras can comprise only the functional portion(s) of the respective domains. For example, in the case of the cytoplasmic domain of FcγRIIA, truncation at amino acid 303 (which results in deletion of the terminal 8 amino acids but preservation of the two tyrosine (Y282 and Y298)-containing core sequences important in phagocytosis does not decrease phagocytosis (Mitchell et al, Clin. Res. 41:1894A

(1993)). Truncation of the FcγRIIA cytoplasmic domain at amino acid 268 or 280, however, results in receptors lacking the tyrosines at positions 282 and 288, and lacking phagocytic activity. These data are consistent with the importance of tyrosine residues in the cytoplasmic Fc receptor domain in transmission of the cytoplasmic signal. In treatment regimens in which suppression of phagocytic potential is advantageous (for example, autoimmune diseases) these later mutants or peptides derived from or mimicking these mutants can be useful (see the commonly owned application entitled "Method of Inhibiting Phagocytosis" filed concurrently herewith, the entire disclosure of which is incorporated herein by reference). It will be appreciated, however, that when potentiation of phagocytosis is sought, functionality of each of the domains must be preserved. In this regard, it appears that the second YX2L of the core sequence of the cytoplasmic domain of FcγRIIA (E-X8-D-X2-Y-X2-L-X12-Y-X2-L) and the γ chain of FcγRIIIA (D/E-X2,7-D/E-Y-X2-L-X7-Y-X2-L) are particularly important for phagocytosis (note also that the exon 3 domain of the γ chain of FcγRIII that is 5' or amino terminal to the Y-X2-L motif appears to play a role in phagocytosis since its elimination diminishes phagocytosis by the γ subunit of FcγRIIIA) (the numbers following the letter X denote the number of amino acids at that position; X can be any amino acid but X within a Y-X2-L preferably represents the amino acids present in the Y-X2-L sequence of the cytoplasmic domain of FcγRIIA or the γ

chain of FcγRIII). Accordingly, it can be expected that phagocytosis can be increased by multiplying the number of copies of the core sequence, for example, in FcγRIIA or in the γ chain of FcγRIIIA, or by multiplying the number of copies of the second Y-X2-L present in those core sequences. The specific amino acids in this second Y-X2-L are important for phagocytosis and appear to provide specificity to the phagocytic signal. It is also expected that phagocytic activity can be increased (as compared to the wild type gamma chain) by, for example, inserting the FcγRIIA second Y-X2-L into the γ chain of FcγRIIIA (as compared to the wild type gamma chain). Furthermore, it is expected that inserting the second cytoplasmic domain Y-X2-L of the γ chain of FcγRIIIA (or both the first and second cytoplasmic domain Y-X2-L of the γ chain) into the ζ chain of FcγRIIIA will increase the phagocytic activity of the ζ chain. Further, the inclusion of two additional Y-X2-L or Y-X3-I motifs to FcγRIIB (which itself is non-phagocytic) renders this receptor phagocytic (this includes adding a variation of the Y-X2-L, Y-X3-I, to the carboxyterminal portion of the cytoplasmic domain). As indicated above, fibroblasts and fibroblast-like cells (for example, COS cells) can be used to assess the operability of a particular receptor construct.

The above-described chimeras of the invention can be constructed by the polymerase chain reaction (PCR) (Horton et al, Biotechniques 8:528 (1990)) using as templates appropriate receptor cDNA and appropriate

oligonucleotides. PCR products can be directly cloned into an expression vector, for example, pSVL, and confirmed by complete sequencing. The expression of the chimeric receptors can be assayed by flow cytometry using anti-Fcγ receptor mAbs and phagocytic function can be evaluated following incubation of IgG-sensitized RBCs.

More specifically, two step overlap extension PCR, a technique that allows introduction of mutations into any part of a PCR fragment, can be used to generate the chimeric molecules of the invention, as well as the mutated/ truncated receptors described herein. In the first step in overlap extension PCR, two primer pairs, 1a and 1b and 2a and 2b, are used to generate two overlapping fragments, 1 and 2. In step 2, when these two fragments are mixed, denatured and reannealed, the 3' end of the sense strand of fragment 1 anneals to the 3' end of the antisense strand of fragment 2. This overlap can be extended to form the entire recombinant product and can be amplified by PCR using primers 1a and 2b. The overlap region is determined by primers 1b and 2a and can contain any sequence as long as parts of the oligomers are complementary. This region is where base changes are incorporated when the technique is used for site directed mutagenesis. Alternatively, the overlap can be designed to make a clean joint between two fragments from two different DNA molecules to form a chimeric molecule. For construction of chimeric mutants, primers 1b and 2a are designed to contain regions from both contributing molecules so that

fragments 1 and 2 can anneal. For example, to construct the chimera containing the FcγRIIIA extracellular region and the transmembrane and cytoplasmic domains of the γ chain, the following 2 pairs of oligomer primers are used (primer 1b is shown 3'-5'):

1a. 5'ACGATGTCTAGAGGTGACTTGTCCACTCC3' (sense)
1b. 3'GGTGGACCCATGGTTGAGACGATATAGGAC5' (antisense)
2a. 5'CCACCTGGGTACCAACTCTGCTATATCCTG3' (sense)
2b. 5'ATGGCGAGCTCTCCGGTAAACAGCATCTGAG3' (antisense)

XbaI and SacI restriction sites can be introduced in primers 1a and 2b respectively so that the final PCR product encoding the chimeric receptor can be ligated in the proper orientation into, for example, an SV40 based expression vector (e.g., PSVL) restricted with XbaI and SacI. To produce truncated molecules, stop codons can be introduced via primers 1b and 2a. In a similar fashion, tyrosine codons can be replaced by phenylalanine codons and serine or threonine codons by alanine codons.

Target cells and modes of administration:

As noted above, the present invention can be used to treat patients that are predisposed to an increased risk of infection. Such patients include, but are not limited to, those suffering from liver disease resulting, for example, from alcoholic cirrhosis, from kidney disorders, such as end-stage renal disease, and from pulmonary disorders including cystic fibrosis and

ARDS. AIDS patients are also appropriate candidates for treatment in accordance with the present invention. In each instance, treatment is effected by increasing the phagocytic potential of cells of the patient.

5 In the case of pulmonary disorders, the receptor-encoding sequence can be administered to the cells of the lung, including macrophages, in the form of an aerosol. The encoding sequence can be present in the aerosol as a particle (e.g. liposome or non-infectious
10 bacteria, for example, Listeria) that is phagocytosed by the pulmonary macrophages. The encoding sequence can also be present in a viral vector.

Viral vectors can also be used to introduce the Fc receptor-encoding sequence of the invention into cells
15 of the pulmonary tree, including fibroblasts, epithelial cells and other cells present in the lung. The vectors can be introduced as an aerosol and can take the form of a replication defective herpes or adenoviral vector. Retroviral vectors can also be
20 used, as well as other viral vectors. (See, generally, Bajocchi et al, Nat. Genet. 3:229 (1993); Lemarchand et al, Circ. Res., 72:1132 (1993); Ram et al, Cancer Res. 53:83 (1993); Crystal, Am. J. Med. 92:44s (1992); Yoshimura et al, Nucl. Acids Res. 20:3233 (1992);
25 Morecki et al, Cancer Immunol. Immunother. 32:342 (1991); Culver et al, Hum. Gene Ther. 1:399 (1990); Culver et al, Transplant. Proc., 23:170 (1991)).

The Fc receptor-encoding sequences of the invention can also be introduced into cells such as T
30 cells thereby rendering them phagocytic. The

advantages of phagocytic T cells are clear, particularly in combating infections that accompany diseases such as AIDS. The abundance of T cells is such that by transforming them with the Fc receptor encoding sequences of the invention, the phagocytic capacity of the blood is substantially increased.

5 T cells can be rendered phagocytic by transforming them in vitro with, for example, a viral vector containing a sequence encoding an Fc receptor (e.g. FcγRIIA). Techniques such as electroporation can also be used. The transformed T cells can then be reintroduced into the patient from which they were derived. Example X details the transformation of T-cells with FcγRIIA and the results presented demonstrate that phagocytic activity is conferred on these cells. In addition, FcγRIIA is phosphorylated in the T-cells when activated, similar to the phosphorylation observed in activated monocytes and macrophages. FcγRIIA activation in these T-cells leads to tyrosine kinase activation and phosphorylation. The T-cell tyrosine kinase ZAP-70 is activated (phosphorylated) upon FcγRIIA activation in T-cells. B lymphocytes are less abundant than T lymphocytes, but they too can be rendered phagocytic using similar protocols (see Example VII).

25 Further, blood monocytes can be transformed ex vivo with the receptor-encoding sequence of the invention (using, for example, physical techniques such as electroporation, or vectors, including viral vectors (e.g., retroviral vectors, adenoviral vectors, or

30

herpes viral vectors); liposomes and Listeria can also be expected to be useful in transforming monocytes and then reintroduced into the patient). This protocol is particularly advantageous when the liver or spleen is the target site.

In addition to the above, the present invention can be used with patients suffering from immune complex diseases such as lupus erythematosus and rheumatoid arthritis to increase local clearance of circulating immune complexes so as to prevent their deposition in tissues, such as the kidney, and in the joints. This increase can be effected by stimulating liver and splenic macrophage phagocytic potential using protocols such as those described herein.

It will be appreciated from a reading of the foregoing that, depending on the target cell and the effect sought, various methods can be used to introduce receptor-encoding sequence into the cell (in addition to electroporation noted above, calcium phosphate as well as other techniques can be used to introduce naked DNA). It will also be appreciated that the gene therapy approach to enhancing phagocytic potential can be used alone or in combination with the drug therapy approach described above. The combination therapy makes it possible to increase the number of naturally occurring receptors and at the same time effect the selective expression of receptors of a particular functional type.

The following non-limiting Examples describe certain aspects of the invention in greater detail.

EXAMPLE I

In Vivo Administration of hrM-CSF Increases
Splenic Macrophage Fc γ Receptors

Human recombinant macrophage colony stimulating factor (hrM-CSF) was studied *in vivo* using an established model in the guinea pig (Schreiber et al, J. Clin. Invest. 51:575 (1972)). Adult male guinea pigs were treated for 5 days with hrM-CSF (500 μ g/kg) and splenic macrophage Fc γ R function and protein expression were assessed by i) the splenic macrophage clearance of IgG sensitized ⁵¹Cr-guinea pig RBC (EA), ii) the *in vitro* binding of EA by isolated splenic macrophage, and iii) FACS analysis using monoclonal antibodies with specificity for the two guinea pig splenic macrophage Fc γ receptors, Fc γ R1,2 and Fc γ R2. Treatment with hrM-CSF enhanced the clearance of EA by 72 \pm 5%. In addition, a greater proportion of isolated splenic macrophages from hrM-CSF treated animals bound EA *in vitro*: 80 \pm 7% vs 48 \pm 4%(sham), $p < 0.001$. *In vivo* hrM-CSF increased the expression of both splenic macrophages Fc γ receptors: 81 \pm 6% and 130 \pm 10% for Fc γ R1,2 and Fc γ R2, respectively. The lowest effective dose of hrM-CSF was 250 μ g/kg, increasing the expression of Fc γ R1,2 by 26 \pm 3% and Fc γ R2 by 42 \pm 4%. At this dose, the clearance of EA was also enhanced. The effect of hrM-CSF required at least 4 days of treatment.

EXAMPLE II

FcγIIA Mediates Phagocytosis and Receptor
Phosphorylation in a Fibroblast Cell Line

Experimental Protocols:5 *Cell culture and reagents:*

 The SAR6 cell line was derived from primary
embryonic mouse fibroblasts in which both Src alleles
had been disrupted by homologous recombination using
the neomycin resistance gene (Thomas et al, Science
10 254:568 (1991)). D58 was derived from primary
embryonic mouse fibroblasts that were wild type for
Src. Cells were maintained in DMEM containing glucose
(4.5 mg/ml), glutamine (25 mg/ml), penicillin (100
U/ml), streptomycin (100 μg/ml) and 10% heat
15 inactivated fetal calf serum.

Retroviral infections:

 FcγRIIA was inserted into the HindIII site of the
retroviral vector pLCX (Miller and Rosman,
Biotechniques 9:908 (1989)) under control fo the CMV
20 promoter. The resulting construct, pLNCX2A, was
transfected into the ecotropic packaging cell line,
Psi2. Two days after transfection, the cells were
diluted 1:20 and G418 resistant colonies were isolated
and assessed for virus production. The stock gave 1 x
25 10 G418 resistant colonies per milliliter. 0.1 ml of
viral stock was used to infect D58 and SAR6 cells (2.5
x 10 cells per infection). Twenty four hours after

infection, the cells were diluted 1:3 and allowed to reach 80-90% confluence before assaying for cell surface expression of FcγRIIA and for phagocytosis. Transient infections were carried out due to the fact that the G418 resistant phenotype of the SAR6 cell line prohibited the selection of stable lines using this retroviral vector.

Flow cytometry:

To determine the extent of FcγRIIA expression on the cell surface of infected D58 and SAR6 cells, samples were stained with fluorescein-labeled anti-FcγRII mAb (IV.3) or with an isotype control (Indik et al, J. Clin. Invest. 88:1766 (1991)). Fluorescence was measured on a FACStar (Becton-Dickinson, Mountainview, CA). 10,000 events were analysed in each case and mean fluorescence intensities were estimated and contour maps were generated using Consort 30 software.

Binding and phagocytosis of IgG-sensitized sheep red blood cells (EA):

EA was prepared as described previously (Indik et al, J. Clin. Invest. 88:1766 (1991)), overlaid onto the infected cells and incubated at 37°C for 30 minutes. Unbound EA was removed by washing with PBS and the plates stained with Wright-Geimsa to assess rosetting. To determine phagocytosis, extracellular bound EA was removed by exposure to hypotonic buffer before staining with Wright-Geimsa.

Biotinylation of cell membranes:

Twenty four hours after infection, FcγRIIA-infected and sham-infected SAR6 and D58 cells were plated on 100 mm petri dishes. After a further twenty four hours, the cells (2×10^6) were washed once with PBS, overlaid with 1.0 ml of PBS containing 100 μ l of 1 M NaHCO₃ and 100 ml of 1 mg/ml biotin (Pierce, Rockford, IL) and incubated at room temperature for 60 minutes. One hundred μ l of NHC1 was added and incubation continued for a further 10 minutes. The cells were washed once with PBS and lysed with 1.0 ml RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10-mM Tris pH7.2, 5 mM NaEGTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM NaVO₃) at 4°C for 30 minutes. FcγRIIA was immunoprecipitated from the biotinylated cell membrane extract with anti FcγRII mAb (Eisman and Bolen, Nature 355:78 (1992)) and analyzed on a 7.5% SDS-polyacrylamide gel (PAGE). Immunoblots were probed with avidin-horseradish peroxidase (BioRad, 1:1000 dilution), followed by Enhanced Chemiluminescence reagents (Amersham Corp.) and visualized using Kodak XAR-5 film.

Phosphotyrosine immunoblots:

FcγRIIA-infected and sham-infected D58 and SAR6 cells (2×10^6 cells per 100 mm petri dish) were overlaid with 500 μ l EA and incubated at 37°C for 30 minutes to activate FcγRIIA. After washing with PBS to remove unbound EA, the bound EA was removed by exposure to hypotonic buffer. Cells were lysed on the plates

with 1.0 ml RIPA buffer at 40°C for 30 minutes and phosphotyrosine containing proteins were immunoprecipitated from the cell lysates using polyclonal rabbit antisera UP28 (Huang et al, J. Biol. Chem. 267:5467 (1992)). The immunoprecipitates were analyzed on a 7.5% SDS-PAGE and immunoblots probed with antiphosphotyrosine mAb, 4G10 (Huang et al, J. Biol. Chem. 267:5467 (1992)).

In vitro immune complex kinase assay of Src-family protein tyrosine kinases from FcγRIIA infected Src- and Src+ cells:

FcγRIIA-infected and sham-infected SAR6 and D58 cells (2 x 10 cells per 100 mm petri dish), were lysed with 1.0 ml RIPA buffer at 4°C, for 30 minutes. Immunoprecipitations were performed by mixing cell lysates with the following mAbs singly or in combination: anti-Src (Lipsiche et al, J. Virol. 48:352 (1983)), anti-FcγRII (Rosenfeld et al, J. Clin. Invest. 76:2317 (1985)), anti-Fyn (Huang et al, J. Biol. Chem. 267:5467 (1992)) and rabbit anti-mouse (RAM) IgG. The immune complexes were incubated with [γ -³²P]ATP to allow autophosphorylation of the kinases and phosphorylation of the substrate and were separated by SDS-PAGE. The gel was washed with 1 N KOH at 55°C for two hours to remove serine/threonine phosphorylation (tyrosine phosphorylation is relatively resistant to alkali) before exposure to Kodak XAR-5 film.

Results of Phagocytosis and Phosphorylation Studies:

Forty eight hours after infection of cell lines D58 and SAR with a retroviral vector containing a FcγIIA encoding sequence, cell surface biotinylation followed by immunoprecipitation with anti-FcγRII mAb demonstrated that the 40 kD receptor was present in the membrane of both Src+ and Src- cells (Figure 1a). Fluorescence histograms of FcγRIIA infected SAR6 and D58 cells are shown in Figure 2. In this representative experiment, sixty five percent of cells expressed the receptor in SAR6 and eighty one percent in D58 with mean fluorescence intensities of ninety five and one hundred and fifty one, respectively. Both Src- and Src+ cells incubated with IgG sensitized cells (EA) bound and phagocytosed these immune complexes. Forty three percent of cells phagocytosed EA in the Src- mutant and seventy percent in D58. In contrast, no binding or phagocytosis was observed in sham infected cells.

To determine if the activated receptor was phosphorylated in the Src- cell line, phosphotyrosine containing proteins were immunoprecipitated from activated and unactivated SAR6 and D58 infected cells. Crosslinking of FcγRIIA with EA resulted in tyrosine phosphorylation of the 40 kD FcγRIIA receptor protein in both Src+ and Src- cells (Figure 1b).

Although Src is not responsible for phosphorylating FcγRIIA in SAR6 cells, FcγRIIA in these mouse fibroblasts was able to act as a substrate for

Src related tyrosine kinases. An *in vitro* immune complex kinase assay was performed on lysates from SAR6 and D58 cells that had been infected with FcγRIIA. Lysates were co-immunoprecipitated with antibodies specific for the receptor protein and with antibodies specific for either Src or Fyn kinases (Figure 3). The co-immunoprecipitates were incubated with [γ ³²P] ATP to allow autophosphorylation of the kinase and phosphorylation of FcγRIIA. FcγRIIA was phosphorylated by Src in this *in vitro* assay (Figure 3, lane 5). Fyn could also phosphorylate FcγRIIA, although to a lesser extent when compared to Src (lane 6). In the absence of the kinases, no phosphorylation of FcγRIIA was observed (lanes 4 and 10) consistent with the lack of tyrosine kinase sequences in the receptor. In the Src-lysates, co-immunoprecipitation with Src and FcγRIIA did not result in phosphorylation of the receptor (lane 11), but a low level of phosphorylation of FcγRIIA was observed in co-immunoprecipitates of FcγRIIA and Fyn (lane 12). This may reflect the efficiency of phosphorylation of the receptor by Fyn; alternatively the fibroblasts may express different amounts of the two kinases.

EXAMPLE III

High Affinity Fcγ Receptor (CD64) Induces Phagocytosis in the Absence of its Cytoplasmic Domain

Wild type (WT) and a mutant (MT) FcγRI, engineered to omit the cytoplasmic domain (CYT), were transfected

into COS cells and murine macrophages (P388D1). The phagocytic potential of the transformed cells was assessed using IgG-coated RBCs (EA) and RBCs conjugated with Fab anti-human FcγR1 mAb (E-mAb). FcγR1, in contrast to FcγRII, did not induce phagocytosis in COS cells (assessed by electron microscopy) but did induce a Ca^{2+} signal which required its CYT. However, both WT and MT FcγRI induced phagocytosis in P388D1. Phagocytosis by WT FcγRI was inhibited by the tyrosine kinase inhibitor tyrphostin 23. Furthermore, activation of FcγRI on monocytes with Fab anti-FcγRI induced tyrosine phosphorylation of FcγRII, determined by anti-phosphotyrosine immunoblots. FcγR1 thus mediates a Ca^{2+} signal through its cytoplasmic domain but not phagocytosis. FcγRI induced phagocytosis therefore requires elements, present in macrophages but absent in COS cells, that permit transmembrane communication.

EXAMPLE IV

20 Structural Requirements of the Human Fc Receptor FcγRIIA in Phagocytosis

25 The structural requirements of FcγRIIA in phagocytosis were examined using COS-1 cells, which lack endogenous Fc receptors, as the recipient in transfection studies. FcγRIIA has two (Y282 and Y298) tyrosine-containing core sequences, Y-X2-L, within a cytoplasmic motif similar to that in other Ig gene family receptors. Truncation of the cytoplasmic domain

at amino acid 268 or 280, to produce mutants lacking both these tyrosines and both core sequences, eliminated phagocytic activity even though these transfectants bound IgG-sensitized cells efficiently.

5 Truncation at amino acid 303, deleting only the terminal 8 amino acid and preserving both core sequences, did not decrease phagocytosis. Substitution of Y282 with phenylalanine (F) inhibited phagocytosis and substitution of Y298 with F partially diminished the phagocytic signal. Substitution with F of the
10 third cytoplasmic tyrosine (Y275) outside the conserved motif did not alter phagocytosis. Replacement of Y282 or Y298 with lysine reduced phagocytosis further, but replacing Y275 with lysine had little effect.

15 Replacement by F of either Y275 or Y298 in combination with Y282 completely eliminated phagocytic function, suggesting that they interact with Y282 in transmission of the signal. In contrast, some phagocytic activity was preserved in mutants containing Y282, but with F at
20 Y275 and Y298. Deletion of T284-L285 within the Y282MTL core sequence also diminished phagocytosis.

The two core Y282-X2-L and Y298-X2-L sequences contain an intervening stretch of amino acids with 2 prolines suggesting an intervening non-helical structure. A
25 mutant, Δ287-291, in which 5 amino acids including the 2 prolines were deleted reduced phagocytic function.

The initial core cytoplasmic sequence Y282MTL and the proline containing region between Y282 and Y298 are important for transmission of the phagocytic signal by
30 FcγRIIA.

EXAMPLE V

The Structure of the γ chain Fc Receptor
Subunit Determines Phagocytic Function of
Macrophage Fc γ RIII (Fc γ RIIIA)

5 A Fc γ RIIIA encoding sequence was transfected into
COS-1 cells to study its phagocytic function,
determined by electron microscopy, in the absence of
other Fc receptors. Co-transfectants of Fc γ RIIIA- α
with either γ or ζ gave equivalent cell surface
10 expression and binding of IgG-coated cells (EA), but γ
was 6 fold more effective than ζ in phagocytosis. To
delineate the region of the γ chain important in
phagocytosis, two deletion mutants, were constructed,
deleting the C-terminal 7 amino acids or deleting the
15 C-terminal 22 amino acids which have a tyrosine
containing conserved motif, Y-X2-L-X7-Y-X2-L, present
in several Ig gene super family receptors. The C-
terminal 7 amino acid deletion demonstrated minimally
reduced phagocytic activity, whereas the more extensive
20 deletion completely eliminated phagocytosis, suggesting
the importance of the conserved cytoplasmic motif. The
role of the conserved cytoplasmic tyrosines was then
examined. Conservative substitution by phenylalnine of
either of the 2 cytoplasmic tyrosines in the γ chain
25 significantly decreased Ca²⁺ signaling and reduced
phagocytosis by >99%. Tyrophostin 23 which alters
tyrosine kinase activity reversibly inhibited
phagocytosis, indicating that phosphorylation of γ
and/or downstream protein tyrosine kinase(s) is

required for a phagocytic signal. Further, single chain Fc γ receptor chimeras, consisting of the γ cytoplasmic domain and the α extracellular domain with the transmembrane domain of either Fc γ RIIIA- γ or Fc γ RI were able to mediate a phagocytic signal. However, single chain chimeras were not sufficient for full phagocytic activity.

EXAMPLE VI

Examination of Phagocytosis by Chimeric Fc γ Receptors

Fc γ RIIA avidly binds and phagocytoses IgG-sensitized cells (EA), as assessed by electron microscopy using the COS cell transfection model system, but Fc γ RI and two other Fc γ RII isoforms, Fc γ RIIB1 and Fc γ RIIB2, do not transmit a phagocytic signal although they also bind EA avidly. Chimeric receptors of Fc γ RI and Fc γ RII were constructed in order to further assess the function of their transmembrane and cytoplasmic domains in phagocytosis. Chimeric transfectants consisting of the extracellular (EC) and transmembrane (TM) regions of Fc γ RIIB2 and the cytoplasmic domain (CYT) of Fc γ RIIA and chimeric transfectants consisting of the EC of Fc γ RI and the TM and CYT of Fc γ RIIA were efficient in phagocytosis. In contrast, phagocytosis was greatly diminished by chimeras consisting of the EC and TM of Fc γ RI and the CYT of Fc γ RIIA. In addition, a chimeric transfectant bearing the EC from Fc γ RI, the TM

from Fc γ RIIA and the CYT from Fc γ RI did not phagocytose EA. These studies indicate that in this system: i) the transmembrane domain of Fc γ RIIB2 is able to provide the necessary structure to permit a phagocytic signal by the cytoplasmic domain of Fc γ RIIA, ii) the transmembrane domain of Fc γ RI is unable to transmit a phagocytic signal to the cytoplasmic domain of Fc γ RIIA, and iii) the transmembrane domain of Fc γ RIIA is unable to confer phagocytic competence to Fc γ RI.

EXAMPLE VII

B-Cell Antigen Receptor Subunit Ig- γ Mediates Phagocytic Signal

The B-cell receptor complex is composed of an antigen recognition subunit noncovalently associated with a membrane subunit consisting of heterodimers of two chains, Ig- α and Ig β/γ , which are products of the mb-1 and B29 genes. Both membrane Ig subunits contain within their cytoplasmic regions a conserved sequence implicated in intracellular signalling. Using COS cell transfectants, the Fc receptor Fc γ RIIA, which is not present in B-cells, has been shown to mediate a phagocytic signal and to contain within its cytoplasmic domain a sequence similar in some aspects to that of Ig- α . Therefore, a Fc γ RIIA and Ig- α chimera was constructed, consisting of the extracellular and transmembrane domains of Fc γ RIIA and the cytoplasmic domain of Ig- α . This chimeric receptor was expressed in COS-1 cell transfectants, determined by flow

cytometry, and bound IgG-sensitized RBCs (EA) efficiently. Furthermore, transfection of this chimeric receptor into COS-1 cells conferred phagocytic competence to COS-1 cells similar in extent to transfection of the receptor FcγRIIA.

EXAMPLE VIII

Alterations in Monocyte/Macrophage Fcγ Receptor Expression in the Acute Respiratory Distress Syndrome (ARDS)

Monocytes from patients with ARDS were used to examine potential alterations in Fcγ receptor expression. Since macrophages may express all 3 classes of Fcγ receptors, specific mAbs for each class of Fcγ receptor and flow cytometry were used to quantitate Fcγ receptor expression. Patients with ARDS met the following four criteria: i) acute bilateral alveolar-type infiltrates on chest radiograph, ii) severe hypoxemic respiratory failure with $\text{PaO}_2/\text{FiO}_2 \leq 150$ without PEEP, iii) absence of congestive heart failure, and iv) having a presumed pre-disposing cause of ARDS. Seven patients with ARDS were compared to 5 normal controls. Whether measured as percent of cells expressing the Fcγ receptor or the difference in mean fluorescence intensity (MFI), FcγRI was reduced in patients with ARDS ($\text{ARDS} = 36.0 \pm 6.3\%$ [mean \pm SEM] or 22.6 ± 7.0 MFI; normal = $52.8 \pm 11.3\%$ or 35.6 ± 6.4 MFI) and FcγRIII was increased ($\text{ARDS} = 15.6 \pm 7.9\%$ or 12.1 ± 4.9 MFI; normals = $0.8 \pm 0.6\%$ or 1.4 ± 1.2 (MFI)). No

correlation was observed between decreased Fc γ RI and increased Fc γ RIII expression, suggesting differential regulation of these receptors *in vivo*. No significant change was observed in the expression of Fc γ RII. Four of seven patients with ARDS died. One patient was restudied following recovery and Fc γ receptors returned to normal values.

EXAMPLE IX

Fc Receptor Defect in Patients with Liver Disease

Experimental Protocols:

Patients:

Forty nine patients (16 women and 33 men) whose mean (\pm SD) age was 55.2 ± 8.3 years were studied. All patients had biopsy proven alcoholic cirrhosis of the liver and were followed up to a minimum period of two years after study: six died within this period. Ten alcoholic non-cirrhotic subjects (4 women and 6 men; age 45 ± 7 years) and, 20 healthy volunteers (6 women and 14 men; age 52 ± 12 years) served as concurrent controls. Patients were classified in three groups according to their degree of liver insufficiency as assessed by the Orrego index (Orrego et al, Gastroenterology 76:105 (1979)).

Study Protocol:

Blood was drawn on admission for the following measurements: (1) blood glucose and urea nitrogen, sodium, potassium, chloride, total calcium, phosphate, magnesium, creatinine, uric acid, total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol, serum aspartate and alanine aminotransferases, gamma-glutamyl transpeptidase, 5'-nucleotidase, alkaline phosphatase, serum protein electrophoresis, complete blood count, prothrombin time, activated partial thromboplastin time, fibrinogen and alpha-fetoprotein; (2) serum IgG, IgA and IgM, determined by radial immunodiffusion (Behring Diagnostics, Madrid); (3) serum C4, determined by hemolytic titration (Gaither et al, J. Immunol. 113:574 (1974)), and serum C3 and C3a desArg, determined by radial immunodiffusion (Behring Diagnostics); (4) plasma levels of zinc, measured by absorption spectrophotometry (pye Unicam SP 190); (5) circulating immune complexes, determined by [¹²⁵I]C1q binding (Zubler and Lamber In: Bloom and David, eds. *In vitro Methods in Cell-Mediated and Tumor Immunity*, New York: Academic Press pp 565-72(1976)); (6) peripheral-smear examination after Wright-Giemsa staining to assess the presence of Howell-Jolly bodies as an index of splenic function (Boyko et al, Am. J. Clin. Pathol. 77:745 (1982)) (negative in all patients); (7) macrophage Fcγ-receptor-dependent clearance *in vivo*; and (8) Fcγ-receptor-mediated recognition of sensitized cells by peripheral-blood monocytes *in vitro*; and (9) abdominal ultrasound to

assess for the presence of splenomegaly, which was detected in 17 out of the 49 patients.

Preparation of human IgG anti-Rh(D):

Human IgG anti-RH(D) was prepared from serum from
5 a single donor (was HIV-1 negative by ELISA-Pasteur
Institute, Madrid-, Western Blott-Pasteur Institute,
Madrid- and the quantitative end-point dilution method)
by ammonium sulfate precipitation followed by Sephacryl
S-300 gel filtration and QAE ion-exchange
10 chromatography (Pharmacia, Madrid). No IgM was
detected by double immunodiffusion (Ouchterlony
analysis). The final IgG fraction was passed through a
Millipore filter and tested for pyrogenicity and
sterility. The final IgG fraction was HIV-1 negative
15 by ELISA (Pasteur Institute, Madrid), Western Blott
(Pasteur Institute, Madrid) and the quantitative end-
point dilution method (Ho et al, N. Engl. J. Med.
321:1621 (1989)).

Macrophage Fcγ-receptor-mediated clearance:

20 Clearance studies were performed as previously
described (Ruiz et al, N. Eng. J. Med. 322:717 (1990);
Frank et al, N. Engl. J. Med. 300:518 (1979); Schreiber
and Frank, J. Clin. Invest. 51:575 (1972)). In brief,
erythrocytes (RhD) were isolated from all subjects,
25 washed three times in physiologic saline,
spectrophotometrically standardized to a concentration
of 6.6×10^6 cells per milliliter, and radiolabeled with
 ^{51}Cr (potassium dichromate, Amersham, Buckinghamshire,

England). An aliquot of cells was sensitized by adding to it drop by drop an appropriate dilution of the purified human IgG anti-Rh(D). The mixture was incubated at 37°C for 30 minutes, and the sensitized ⁵¹Cr-labeled erythrocytes were washed four times in saline and resuspended to a concentration of 3.3x10 per milliliter in Hanks' balanced salt solution (M.A. Bioproducts, Madrid). An aliquot of cells (usually 10 ml, with 2.5 µCi of radioactivity) was injected through an antecubital vein, and the survival of red cells was determined in serial blood samples obtained over a period of 48 hours. Clearance curves were plotted by expressing the number of counts per minute at each time point as a percentage of the number of counts at 10 minutes, the zero point. The time required for clearance of the 50 percent of the inoculated IgG-coated red cells (half-time) was calculated and then correlated with clinical and serologic data. In addition, for the clearance on each day, the percentage for the inhibition of clearance above control was calculated at 1, 1.5, 2, 8, 24 and 48 hours, according to the formula

$$\% \text{ inhibition} = 100 \times 1 - \frac{(\text{CPMb} - \text{CPMx})}{(\text{CPMb} - \text{CPMc})},$$

where CPMb denotes the number of counts per minute in a control subject who received an injection of unsensitized autologous red cells, CPMx the number of counts in a patient who received IgG-coated (sensitized) autologous red cells, and CPMc the number

of counts in a control subject who received autologous IgG-sensitized red cells. By means of this formula, patients could be compared with controls studied on the same day, and results could be expressed as the percentage of change in clearance, where 100 percent inhibition of clearance indicated that clearance in a patient who received IgG-coated red cells (CPMx) was identical to clearance in a control who received unsensitized red cells (CPMb) (Friedman, J. Clin. Invest. 75:162 (1985)). In three additional control groups - five patients with alcoholic cirrhosis of the liver, five non-cirrhotic alcoholic subjects, and five healthy volunteers - the clearance of autologous ⁵¹Cr-labeled but unsensitized red cells and the clearance of ⁵¹Cr-labeled heat-damaged autologous red cells were examined.

Duplicate studies were performed in nine of the patients with alcoholic cirrhosis of the liver in whom severe infection had developed, six of the patients with alcoholic cirrhosis of the liver without a history of complications due to infection, and six controls. The results of the repeat studies of clearance were unchanged from those of the original studies in each subject. Serum C3, C3a desArg, and C4 were measured to assess complement activation during the clearance of IgG-coated red cells. No significant complement activation was observed in any of the patients included in the present study.

Number of IgG (Anti-RhD) molecules per red cell:

The number of IgG molecules per red cell was determined as previously described with the use of ¹²⁵I-labeled anti-IgG reagent (Cines and Schreiber, N. Engl. J. Med. 300:106 (1979)). Clearance studies were always performed with erythrocytes sensitized so that approximately 600 molecules of IgG were present on each red cell. When Fcγ-receptor-dependent recognition by blood monocytes was studied *in vitro*, each red cell (RhD) was coated with 400, 800, or 1600 molecules of IgG.

Binding of IgG(Anti-RhD)-coated red cells:

The recognition of IgG-coated red cells (RhD) by monocytes isolated was determined as previously described (Gomez et al, J. Reticuloendothel. Soc. 31:24 (1982); Schreiber et al, J. Clin. Invest. 56:1189 (1975)). In brief, confluent monolayers of 5.5×10^5 monocytes were obtained from defibrinated blood after density-gradient centrifugation (Ficoll-Isopaque) and plastic adherence to petri dishes (Nunc, Amsterdam). An aliquot of 2×10^7 ⁵¹Cr-labeled, IgG-coated red cells (RhD) was added to each monocyte monolayer. The petri dishes were then incubated at 37°C in an atmosphere of 5 percent carbon dioxide for 45 minutes, washed to detach unbound red cells, and treated with 0.086 M EDTA solution to remove adherent monocytes and monocyte-bound IgG (Anti-RhD)-sensitized red cells. The treatment with EDTA removed all adherent monocytes and all radioactivity. The percentage of

⁵¹Cr-labeled and IgG-sensitized red cells (RhD) recognized by peripheral-blood monocytes was determined according to the formula:

$$\% \text{red-cell IgG bound to monocyte monolayers} = \frac{\text{cpm for IgG (anti-RhD)-coated red cells removed with EDTA}}{\text{cpm for IgG (antiRhD)-coated red cells added to monocyte monolayers}} \times 100.$$

No phagocytosis of anti-RhD-sensitized erythrocytes by peripheral blood monocytes occurs under the experimental conditions (Gomez et al, J. Reticuloendothel. Soc. 31:241 (1982); Schreiber et al, J. Clin. Invest. 56:1189 (1975)). The studies were repeated in 6 controls, 6 non-cirrhotic alcoholic patients, 9 of the patients in whom severe infection developed and 6 of the patients with no history of infectious complications; the results of the repeat studies were unchanged from those of the original studies in each subject.

Preparation of IgG2b-sensitized red cells:

Antibody-sensitized sheep erythrocytes (EA) were prepared as previously described (Rossman et al, Exper. Hematol. 21:177 (1993)). In brief, 1×10^8 sheep red blood cells in 1.0 ml of 0.01 mol/L EDTA buffer were

sensitized by adding mouse monoclonal antibody Sp2/HL, subclass IgG2b (Serotec Ltd., Bicester, Oxon), in 0.1 ml at 37°C for 1 hour. The final antibody dilutions used to prepare these cells were between 1:10 and 1:80. The IgG-sensitized (coated) sheep red cells were washed twice and resuspended in HBSS to a final concentration of 1×10^6 cells/ml. In addition, a polyclonal 7S IgG rabbit anti-sheep red blood cell (Cordis Laboratories) was also used to prepare polyclonal IgG-coated red blood cells. The final antibody dilution used to prepare these cells was 1:1000.

Monocyte recognition of sheep IgG-sensitized red blood cells:

Monocyte in vitro recognition of IgG-sensitized red cells was assessed as previously reported (Rossman et al, Exper. Hematol. 21:177 (1993); Schreiber et al, N. Engl. J. Med. 316:503 (1987)). In brief, 1×10^6 IgG-coated red cells or control unsensitized red cells were added to monocyte monolayers containing 1×10^6 cells. These cells were incubated at 4°C or 37°C in phosphate buffer at an ionic strength of $\mu = 0.07$ or $\mu = 0.15$, respectively. After 2 hours, the plates were washed and stained with Wright's Giemsa. Two hundred (200) monocytes were counted under light microscopy in a blinded fashion to assess the number of IgG-sensitized red blood cells bound per monocyte. Monocytes binding >3 red blood cells/monocyte were determined. These experiments were performed in 5 patients of each alcoholic cirrhosis of the liver groups (I, II and, III), 5 alcoholic non-cirrhotic

subjects and 5 normal volunteers. The experiments were repeated in these same patients and controls at least one year after the initial studies. No significant variations were found between the initial experiments and the ones performed after more than one year.

HLA typing:

HLA typing was performed by the tissue-typing laboratory of the Virgen del Rocio University Hospital, Seville, Spain.

Assessment of nutritional status:

Nutritional status was evaluated according to anthropometric, biochemical, and immunologic measurements (Blumonkrantz et al, Am. J. Clin. Nutr. 33:1567 (1989); Harvey et al, Am. J. Clin. Nutr. 33:1587 (1989); Feliffe, Wo 1966, No. 53, Geneva, Switzerland; Bristian et al, JAMA 235:1567 (1976)). Dry body weight, relative body weight, and the percent ideal body weight were also determined. The anthropometric data were compared with standard values for the local population (Jaurrieta, Med. Clin. 81:584 (1983)). Serum albumin and transferrin were measured to evaluate the serum protein level. Malnutrition was classified according to previously established criteria (Blumenkrantz et al, Am. J. Clin. Nutr. 33:1567 (1980); Harvey et al, Am. J. Clin. Nutr. 33:1586 (1980); Feliffe, WO 1966 No. 53, Geneva, Switzerland; Bristian et al, JAMA 235:1567 (1976); Jaurrieta, Med. Clin. 81:584 (1983); O'Keefe et al, Lancet 2:615 (1980)) as

marasmus, kwashiorkor, or mixed type. All malnourished patients had malnutrition of the mixed type. A high incidence of protein-calorie malnutrition of the mixed type was observed in 17 of the 49 patients (35 percent). Total body weight did not change. Cutaneous hypersensitivity responses to standard concentrations of four antigens-purified protein derivative, *Trycophyton rubrum*, *Candida albicans*, and streptokinase-streptodornase- were used to evaluate cell-mediated immunity as previously described (Harvey et al, Am. J. Clin. Nutr. 33:1586 (1980); Blackburn et al, J. Parenter. Enteral. Nutr. 1:11 (1977)). A response was considered positive when the diameter of induration was more than 5 mm. A normal response was indicated by a positive response to either three or four antigens, an abnormally low response by a positive response to either one or two antigens, and anergy by a lack of positive response to any of the four antigens.

Statistical analyses:

The *in vivo* clearance curves were analyzed at the time points to calculate a P value for the difference between the controls and patients by Student's t-test. The *in vitro* Fcγ-receptor-dependent recognition of red cells by monocytes and the clearances in patients and controls were assessed with the Wilcoxon rank-sum test for unpaired data. The relation of the clearance rate (as half-time) or monocyte Fcγ-receptor-dependent recognition of IgG-coated red cells *in vitro* to the

seologic tests was analyzed with the Spearman rank-correlation test.

Clearance Study Results:

5 Clearance studies were performed in the 49 patients with alcoholic cirrhosis of the liver fulfilling the inclusion criteria of this study. The results demonstrated that the clearance of IgG-coated red cells was significantly impaired ($p < 0.001$) (Figure 4). At 1 and 1.5 hours, the mean (\pm SEM) inhibition of
10 macrophage Fc γ -receptor-mediated clearance was 47 ± 3 and, 53 ± 3 percent, respectively. Clearance was inhibited by more than 15 percent in 37 patients and, by 5 to 12 percent in 6. In contrast, the clearance of
15 unsensitized red cells and of heat-damaged red cells in the patients did not differ from the clearance of these cells in the non-cirrhotic alcoholics and healthy volunteers (Figure 4).

Patients were classified in three groups according to their degree of liver insufficiency as assessed by
20 the Orrego index. Clearance studies of those three groups of patients are represented in Figure 5. The results demonstrated that the clearance of IgG-coated red cells was significantly impaired ($p < 0.001$) in patients with moderate (Patients II or group II) and
25 severe (Patients III or group III) liver insufficiency. At 1 and 1.5 hours, the mean (\pm SEM) inhibition of macrophage Fc γ -receptor-mediated clearance was 47 ± 3 percent and 66 ± 4 percent, respectively, for group II

patients. At 1 and 1.5 hours the mean (\pm SEM) inhibition of macrophage Fc γ -receptor-mediated clearance of IgG-coated red cells was impaired in patients with mild liver insufficiency (Patients I or group I), (Figure 5), but the difference was not significant.

The patients were followed up for at least two years after the clearance studies were initially performed. Six patients died, two of massive hemorrhage from ruptured esophageal/gastric varices (15th and 17th month of follow up, respectively), two spontaneous bacterial peritonitis by *E. coli* (14th and 20th month of follow up, respectively), and two Gram-negative sepsis due to *E. coli* and (16th and 21st month of follow up, respectively). Eleven patients had severe infection: five had spontaneous bacterial peritonitis (*E. coli*) and, six had sepsis (due to *E. coli* in three, *Staphylococcus aureus* in one, in one, and *Serratia marcescens* in one). When the clearance of IgG-coated red cells in the patients with severe infection was compared with the clearance in the patients without infection, those with infection were found to have a significantly longer half-time (126.2 ± 22 vs. 32.2 ± 18 hours; $p < 0.001$) (Figure 6). The clearance of IgG-coated red cells was analyzed in the patients (half-time) in relation to various parameters of liver impairment (SGOT, SGPT, GGT, 5'-nucleotidase, bilirubin -total, direct and indirect-, P.T., aPTT, fibrinogen and serum albumin). None of these parameters, including the presence of splenomegaly,

correlated with the extent of impairment of clearance of IgG-coated red cells.

Isolated peripheral blood monocytes were also studied (Figure 7). Erythrocytes from a single Rh(D)-positive donor were sensitized with three different concentrations of IgG-antiRh(D) (400, 800, and 1600 IgG molecules per red cell). Monocytes isolated from the patients bound fewer IgG-coated red cells than did those from the controls, but the difference was not significant. There was no correlation between the extent of binding by monocytes and the degree of impairment of clearance of IgG-coated red cells. No difference was observed between this alteration in monocyte FcγRI in patients in whom severe infection developed and those in whom it did not.

The function of monocyte FcγRII was assessed in vitro by the binding of IgG2b-coated red blood cells (Figure 8). Peripheral blood monocytes isolated from patients with cirrhosis of the liver bound less IgG2b-sensitized red cells than monocytes from non-cirrhotic alcoholic subjects or monocytes from normal volunteers, but the difference was not significant.

Seven patients had elevated levels of circulating immune complexes. The clearance of IgG-coated red cells in these patients did not differ from that observed in the patients in general (Figure 9). Furthermore, there was no correlation in these five patients between the level of circulating immune complexes and the extent of impairment of the recognition of IgG-coated red cells by monocytes.

Neither the clearance of IgG-sensitized erythrocytes, nor the recognition *in vitro* of IgG-coated red cells or IgG2b-coated red cells by monocytes from the patients correlated with their sex, age, time from diagnosis of alcoholic cirrhosis of the liver or with any of the serologic measurements, including the immunoglobulin level. Furthermore, there was no relation between either the clearance of IgG-coated red cells or their recognition *in vitro* by monocytes and the HLA haplotype, or the nutritional status of the population studied.

The plasma zinc level was 18.4 ± 0.7 μmol per liter (120 μg per deciliter) in healthy volunteers and 12.7 ± 1.3 μmol per liter (83.3 ± 3.7 μg per deciliter) in the patients with alcoholic cirrhosis of the liver ($p < 0.001$). However, there was no correlation between the plasma zinc level and the degree of impairment of clearance *in vivo* or the monocyte recognition of IgG-coated red cells *in vitro*. Similarly, malnutrition was not necessarily linked with greater impairment of the clearance rate or a lower value for *in vitro* monocyte recognition of IgG-sensitized red cells. The prevalence of malnutrition was significantly higher in the patients with either moderate or severe liver insufficiency (groups II and II, respectively) ($p < 0.001$). However, neither the macrophage Fc γ -receptor-mediated clearance nor the binding of IgG (Anti-RhD)-coated red cells or the binding of IgG2b-coated red cells by monocytes correlated with the

nutritional status of these patients, as indicated by anthropometric, biochemical, and immunologic values.

EXAMPLE X

T-Cells Transfected with FcγRIIA

5 Experimental Protocols:

Cell lines and antibodies:

The Jurkat T-cell line J32 and the CD2-CD28-CD3+ variant J32-3.2 have been described previously (Makni et al, J. Immunol. 146:2522 (1991) and Sancho et al, J. Immunol. 150:3230 (1993)). These cell lines were maintained in RPMI 1640 containing 10% heat inactivated FCS (Hyclone Laboratories, UT), 2mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 U/ml). The following antibodies were used in this study: anti-CD2 mAbs 9.6 (Sancho et al, J. Immunol. 150:3230 (1993)) and 9.1 (Yang et al J. Immunol. 137:1097 (1986)), anti-CD3 mAb 64.1 (Hansen et al, In Leukocyte Typing, Bernard et al eds. Springer-Verlag, New York p. 195 (1984)) and anti-FcγRII mAb IV.3 (Fanger et al, Immunol. Today 10:92 (1989)).

Construction of the FcγRIIA expression vector and DNA transfer into J32 and J32-3.2 cell lines:

FcγRIIA cDNA was isolated from the plasmid pKC4 (Hibbs et al, Proc. Natl. Acad. Sci. USA 5:2240 (1988)) using EcoR1 and the fragment was blunt ended using

Klenow polymerase. The Fc γ RIIA cDNA was then inserted into the SmaI site of plasmid pGSE1731 (Greaves et al, Cell 56:979 (1989)) under control of the human β -globin gene promoter and enhancer sequences. pGSE1731 contains 4.9 Kb of the human β -globin gene including 1.5 Kb of sequences upstream of the CAP site and the internal and 3' enhancer regions. This plasmid also contains the CD2 3' enhancer region which confers T-cell specific, position-independent gene expression (Greaves et al, Cell 56:979 (1989)). The resulting plasmid, pGSE2A was introduced into the J32 and J32-3.2 cell lines by electroporation using methods previously described in detail (Sancho et al J. Immunol. 150:3230 (1993)). Prior to electroporation, pGSE2A was linearized by digestion with NotI. Each electroporation was carried out using 30 μ g of linearized pGSE2A and 5 μ g of pcEXV Neo linearized with EcoRI. After electroporation, the cells were cultured for seven days in the presence of 0.3 mg/ml G418 and assayed for Fc γ RIIA expression by flow cytometry. Fc γ RIIA expressing cells were enriched by immunomagnetic positive selection using magnetic particles coated with IgG (Dynal Inc., Fort Lee, NJ). Cells were cultured in flat bottomed microtitre wells (approx. 100 cells per well) and clones were selected and analyzed for Fc γ RIIA expression by flow cytometry.

* *

Tyrosine Phosphorylation Results:

Stimulation of the T-cell receptor (TCR)/CD3 complex in Jurkat T-cells induces the tyrosine phosphorylation of proteins including the TCR-associated ζ chain, the ZAP70 tyrosine kinase and the CD3 ϵ complex (Weiss, Cell 79:209 (1993)). Similarly, in the members of the IgG family of receptors, induction of tyrosine phosphorylation accompanies receptor activation (Samelson and Klausner, J. Biol. Chem. 267:24913 (1992)) and, accordingly, studies were conducted to determine if stimulation of Fc γ RIIA in the T-cell transfectants J32/Fc γ RIIA and J32-3.2/Fc γ RIIA induced tyrosine phosphorylation. The mutant J32-3.2 cell line is deficient in the induction of tyrosine phosphorylation signalling pathways leading to impaired induction of phosphorylated ZAP70, ζ chain and CD3 ϵ after TCR crosslinking (Sancho et al, J. Immunol. 150:3230 (1993)). The activation of the Src-related tyrosine kinase (SRTKs) p56lck and p59fyn is also defective in this mutant (Sancho et al, J. Immunol. 150:3230 (1993)).

Stimulation of Fc γ RIIA by crosslinking with anti-Fc γ RII antibody followed by immunoprecipitation with anti-phosphotyrosine antibody (Huange et al, J. Biol. Chem. 267:5467 (1992)), showed that the 40 kD Fc γ RII receptor is phosphorylated on tyrosines in both wild-type J32 and in the mutant J32-3.2 transfectants (Figure 10, lanes 4-11 (the position of the 40 kD receptor is indicated with an arrow)).

Phagocytosis Results:

FcγRIIA cDNA was expressed in the wild type Jurkat T-cell line J32 and in the mutagenized J32 variant, J32-3.2. As noted above, the J32-3.2 cell line is CD2-CD28-CD3+ and exhibits reduced signal transduction capabilities after TCR/CD3 stimulation, with respect to tyrosine phosphorylation pathways and GTP binding mechanism (Sancho et al, J. Immunol. 150:3230 (1993)). Calcium mobilization and IL2 promoter activity induced after TCR stimulation are also impaired (Sancho et al, J. Immunol. 150:3230 (1993)). Fluorescence histograms of J32/FcγRIIA and J32-3.2/FcγRIIA stable transfectants, and FcγRIIA expressing clones isolated from these transfected cells, are shown in Figure 11.

The ability of these T-cell transfectants to phagocytose IgG-sensitized cells was assessed by incubation with IgG coated sheep erythrocytes (sEA). In both the wild type J32 and mutant J32-3.2 transfectants, a number of the cells were able to phagocytose the sEA (Figure 12). The results of several experiments with (a) bulk cell stable FcγRIIA-transfectants and (b) FcγRIIA clones are shown in Table 1. The data indicate that these T-cell transfectants phagocytose EA and that phagocytosis by the J32-3.2 mutant transfectants was reduced compared to the wild type cells.

TABLE 1

Phagocytosis of Sheep EA by FcγRIIA bulk cell
stable transfectants of J32 and J32-3.2 cell lines.

5	J32 / FcγRIIA			J32-3.2 / FcγRIIA		
	%	P1		%	P1	
	cP1					
	1.	17	28	-	-	-
	2.	33	22	5	6	7
	3.	25	40	13	18	21
10	4.	32	53	17	21	24
	5.	31	51	-	-	-
	6.	14	21	6	8	9

P1 is the phagocytic index, i.e., the number of
erythrocytes ingested per 100 cells. The corrected P1
15 value (eR1) is included in the J32-3.2 / FcγRIIA column
to take into account the lower MFI value observed in
these transfected cells compared to the J32/FcγRIIA
transfected cells. % = % phagocytic cells.

20 Considering that 70%-100% of the cells are
expressing FcγRIIA in these transfectants, and
presumably are mediating phagocytosis through this
receptor, the levels of phagocytosis observed are
relatively low when compared, for example, to COS-1

fibroblasts transfected with Fc γ RIIA (Indik et al, J. Clin. Invest. 88:1766 (1991)). However, the ingestion of the erythrocytes appears to be mediated via a genuine phagocytic process as preincubation of the cells in 10 μ g/ml cytochalasin-D, a compound which inhibits actin polymerization (a process that is necessary for phagocytosis) (Indik et al, J. Clin. Invest. 88:1766 (1991)), abolished phagocytosis in these cells. Also phagocytosis was inhibited when the transfectants were incubated with sEA at 0°C instead of 37°C.

EXAMPLE XI

Induction of Phagocytosis by a Protein Tyrosine Kinase

Using the COS-1 cell experimental model to define the structural requirements for phagocytosis, it has been established that isoforms of each of the three classes of the Fc γ receptors Fc γ RI, Fc γ RII and Fc γ RIII, are able to transmit a phagocytic signal in transfected COS-1 cells and that both Fc γ RI and Fc γ RIIIA require the γ subunit for this signalling event. To determine the *in vivo* kinase important for γ chain mediated phagocytosis, the monocyte/macrophage protein tyrosine kinase Syk, shown to be associated with the γ chain in monocytes and macrophages, was co-transfected (Yagi et al, Biochem. Biophys. Res. Commun. 200:28 (1994)). The expression vectors used were as follows: Syk (full length cDNA) - pME18S; Fc γ RI - pKC4; Fc γ RIIIA - pSVL;

and γ - pSVL. Syk dramatically enhanced phagocytosis mediated by both Fc γ RI/ γ and Fc γ RIIIA/ γ (Fc γ RI/ γ , 8.0 ± 2.0 fold; Fc γ RIIIA/ γ , 6.1 ± 0.6 fold) and, in addition, increased the number of cells able to mediate phagocytosis (Fc γ RI/ γ , 3.6 ± 0.5 fold; Fc γ RIIIA/ γ , 3.0 ± 0.2 fold). Two γ chain cytoplasmic YXXL sequences were required but neither the cytoplasmic domain of Fc γ RI nor Fc γ RIIIA was necessary for the Syk effect. Syk expression also enhanced (5-7 fold) Fc γ RI and Fc γ RIIIA phagocytosis mediated by the ξ chain, a subunit homologous to γ , but did not increase the level of phagocytosis to that observed for the γ chain. The action of Syk was less pronounced (1.5 ± 0.2 fold) for the phagocytic Fc γ RII receptor, Fc γ RIIA, which does not require the γ chain for phagocytosis. However, Syk stimulated phagocytosis (6.0 ± 1.0 fold) by the poorly phagocytic Fc γ RII receptor Fc γ RIIB2, which contains only a single YXXL sequence, when an additional tyrosine containing sequence, YMTL, was introduced. No enhancement of Fc γ RI/ γ or Fc γ RIIIA/ γ mediated phagocytosis was observed when Fyn, a protein tyrosine kinase of the Src family which is also expressed in monocyte/macrophages, was co-transfected with Fc γ RI/ γ or Fc γ RIIIA/ γ . Similarly, no enhancement was observed when the protein tyrosine kinase ZAP-70, of the Syk family of kinases, was used. These findings indicate that there is specificity of Syk for γ chain sequences.

* * * *

All documents cited hereinabove are incorporated in their entirety by reference.

5 While the invention has been described with respect to what is presently regarded as the most practical embodiments thereof, it will be understood by those of ordinary skill in the art that various alterations and modifications may be made which nevertheless remain within the scope of the invention as defined by the claims which follow.

WHAT IS CLAIMED IS:

1. A method of increasing the phagocytic potential of cells present in a mammal comprising introducing into said cells a DNA molecule coding for an Fc receptor under conditions such that said DNA molecule is expressed and said Fc receptor thereby produced and the phagocytic potential of said cells thereby increased.
2. A method according to claim 1 wherein said cells are phagocytic prior to introduction of said DNA molecule.
3. The method according to claim 1 wherein said cells are macrophages.
4. The method according to claim 1 wherein said cells are monocytes.
5. The method according to claim 1 wherein said cells are non-phagocytic prior to introduction of said DNA molecule.
6. The method according to claim 5 wherein said cells are fibroblasts.
7. The method according to claim 5 wherein said cells are T cells or B cells.

8. The method according to claim 1 wherein said cells are lung cells.

9. The method according to claim 1 wherein said DNA molecule is present as an insert in a viral vector.

10. The method according to claim 1 wherein said DNA molecule is present in a liposome.

11. The method according to claim 1 wherein said DNA molecule is present in a bacterium.

12. The method according to claim 1 further comprising contacting said cells with an effective amount of a drug that increases the phagocytic potential of said cells.

13. The method according to claim 12 wherein said drug is γ -interferon, an estrogen or estrogen analog, M-CSF or GM-CSF.

14. A method of increasing the phagocytic potential of cells of a mammal comprising:

- i) removing cells from said mammal,
- ii) introducing into said cells a DNA molecule encoding an Fc receptor, and
- iii) reintroducing said cells into said mammal under conditions such that said DNA molecule is expressed and said Fc receptor thereby produced and the phagocytic potential of said cells thereby increased.

15. The method according to claim 14 wherein said cells are T cells or B cells.

16. The method according to claim 14 further comprising contacting said cells with an effective amount of a drug that increases the phagocytic potential of said cells.

17. The method according to claim 16 wherein said drug is γ -interferon, an estrogen or estrogen analog, M-CSF or GM-CSF.

18. A liposome comprising a DNA molecule encoding an Fc receptor.

19. A bacterium comprising a DNA molecule encoding an Fc receptor.

20. A T cell comprising an exogenous DNA sequence encoding an Fc receptor.

21. A B cell comprising an exogenous DNA sequence encoding an Fc receptor.

22. A DNA construct encoding an Fc receptor comprising domains, or functional portions thereof, from at least two of Fc γ RI, Fc γ RII, the α chain of Fc γ RIII and the γ chain of Fc γ RIII, wherein said domains, or portions thereof, are such that said Fc receptor renders phagocytic a cell comprising same.

23. The DNA construct according to claim 22 wherein said construct encodes the extracellular domain of the α chain of Fc γ RIIA, the transmembrane domain of the γ chain of Fc γ RIIA or Fc γ RI and the cytoplasmic domain of the γ chain of Fc γ RIIA.

24. The DNA construct according to claim 22 wherein said construct comprises, in a cytoplasmic domain encoding portion thereof, at least two sequences encoding Y-X2-L, wherein X2 represents any two amino acids.

25. The DNA construct according to claim 24 wherein X2 represents the amino acids of a Y-X2-L sequence of the cytoplasmic domain of Fc γ RIIA or the γ chain of Fc γ RIII.

26. A cell comprising the construct according to claim 22.

27. An Fc receptor comprising domains, or functional portions thereof, from at least two of Fc γ RI, Fc γ RII, the α chain of Fc γ RIII and the γ chain of Fc γ RIII, wherein said domains, or portions thereof, are such that said Fc receptor renders phagocytic a cell comprising same.

28. The receptor according to claim 27 wherein said receptor comprises, in a cytoplasmic domain

thereof, at least two copies of the sequence Y-X2-L, wherein X2 represents any two amino acids.

29. The receptor according to claim 28 wherein X2 represents the amino acids of a Y-X2-L sequence of the cytoplasmic domain of FcγRIIA or the γ chain of FcγRIII.

30. An Fc receptor comprising, in a cytoplasmic domain thereof, at least one copy of the sequence Y-X2-L in excess of the corresponding naturally occurring cytoplasmic domain, wherein X2 represents any two amino acids.

31. The Fc receptor according to claim 30 wherein X2 represents the amino acids of a Y-X2-L sequence of the cytoplasmic domain of FcγRIIA or the γ chain of FcγRIII.

32. A DNA sequence encoding the Fc receptor according to claim 30.

33. An Fc receptor comprising, in a cytoplasmic domain thereof, at least one repeat of a Y-X2-L sequence, wherein X2 represents any two amino acids.

34. The Fc receptor according to claim 33 wherein X2 represents the amino acids of a Y-X2-L sequence of the cytoplasmic domain of FcγRIIA or the γ chain of FcγRIII.

35. An Fc receptor comprising, in a cytoplasmic domain thereof, one or more repeats of an FcγRIIA or γ chain FcγRIIIA or FcεRI cytoplasmic domain or portion thereof.

36. The receptor according to claim 35 wherein said portion includes Y-X2-L, wherein X2 represents the amino acids of a Y-X2-L sequence of the cytoplasmic domain of FcγRIIA or the γ chain of FcγRIII or of FcεRI.

37. A method of treating an infection comprising administering to a mammal in need of such treatment a DNA molecule encoding an Fc receptor,

wherein said administration is effected under conditions such that said DNA molecule is expressed in the cells of said mammal, said Fc receptor is produced, and the phagocytic potential of said cells thereby increased, and

wherein said cells phagocytose particles causing said infection.

38. The method according to claim 37 wherein said infection is present in the lungs of said mammal and said cells are lung cells.

39. The method according to claim 37 wherein said cells are liver cells.

40. The method according to claim 37 wherein said cells are spleen cells.

41. The method according to claim 37 wherein said cells are T cells or B cells.

42. The method according to claim 37 further comprising administering to said mammal a drug that increases the phagocytic potential of said cells.

43. The method according to claim 42 wherein said drug is γ -interferon, an estrogen or estrogen analog, M-CSF or GM-CSF.

44. A method of stimulating the phagocytic potential of a cell comprising introducing into said cell a construct comprising a nucleotide sequence encoding the Syk gene under conditions such that said Syk gene is expressed and phosphorylation of Fc γ receptors in said cell is effected.

45. The method according to claim 44 wherein the phagocytic potential of said cell derives from a naturally occurring Fc γ receptor.

46. The method according to claim 44 further comprising introducing into said cell a construct comprising a sequence encoding an Fc γ receptor.

Fig. 1B

B. Phosphotyrosine induction

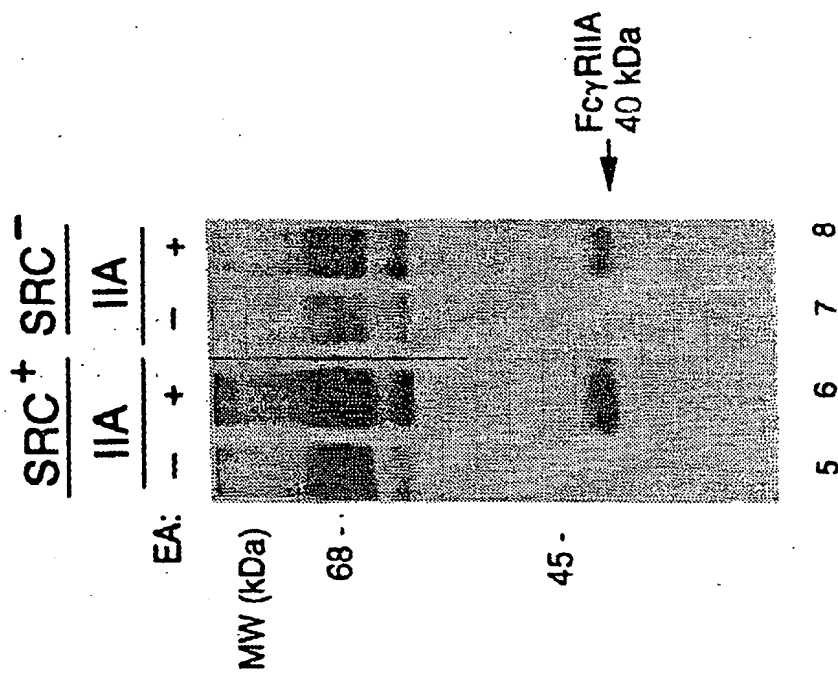
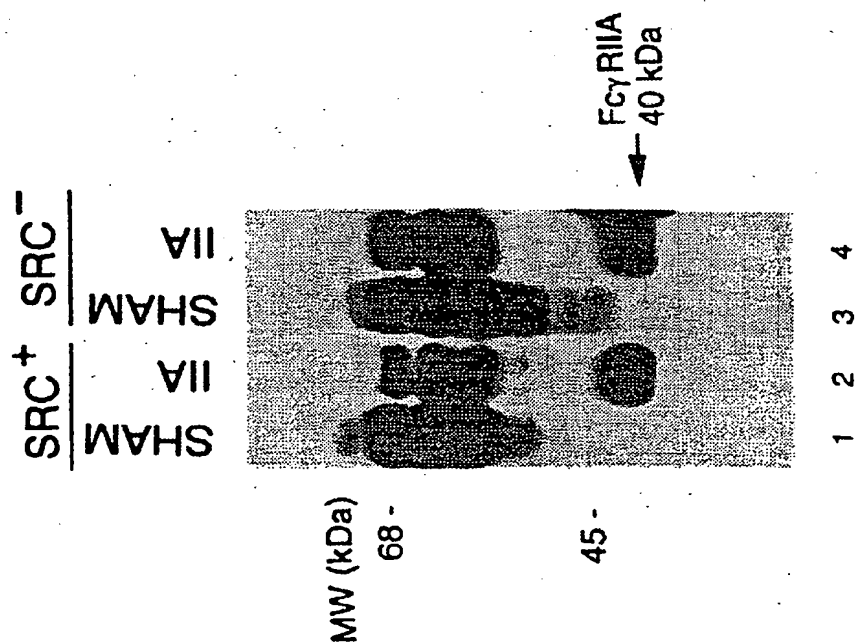
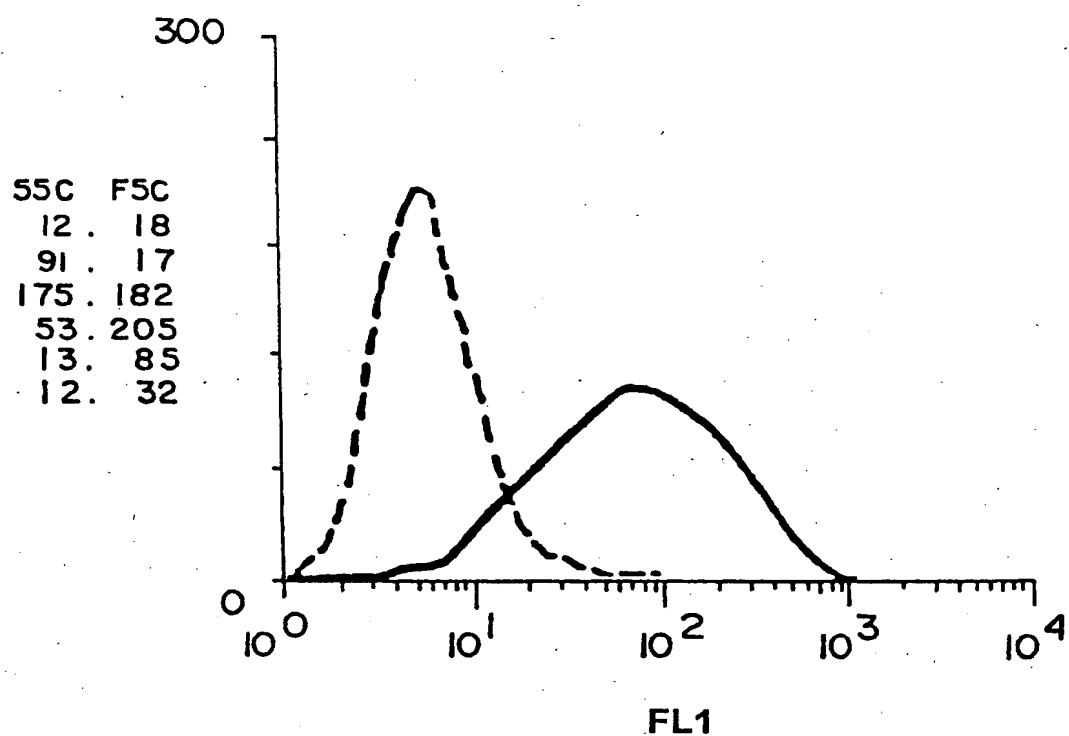
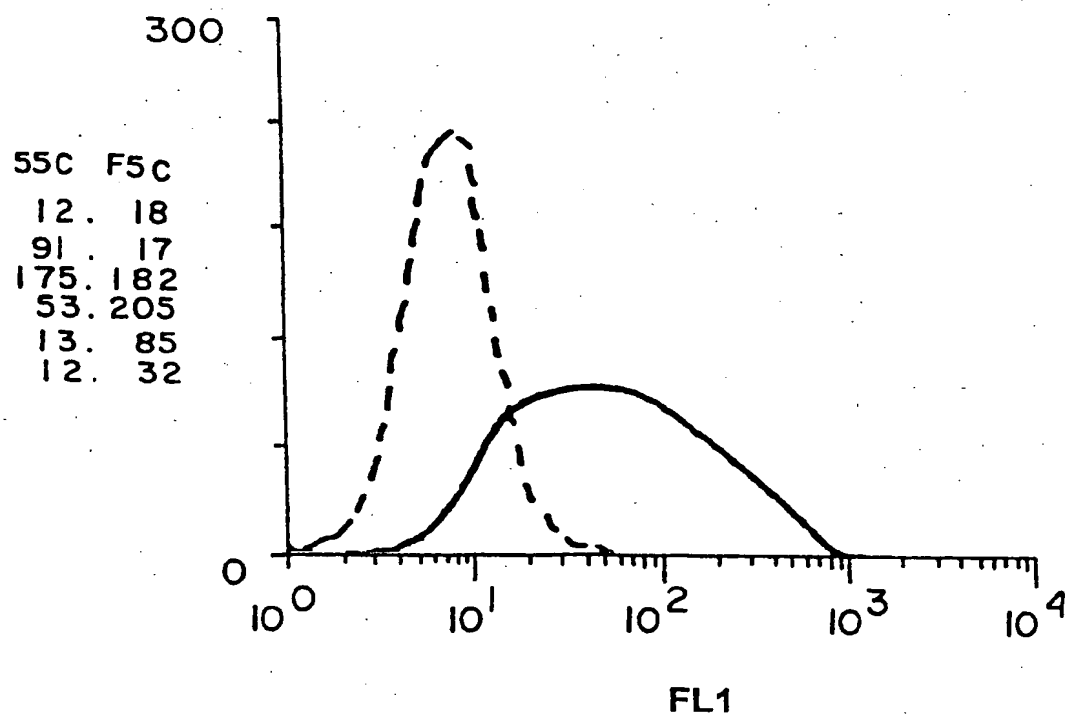


Fig. 1A

A. Biotinylation of membrane



2 / 1 2

Fig. 2A**Fig. 2B**

3 / 1 2

Fig. 3B

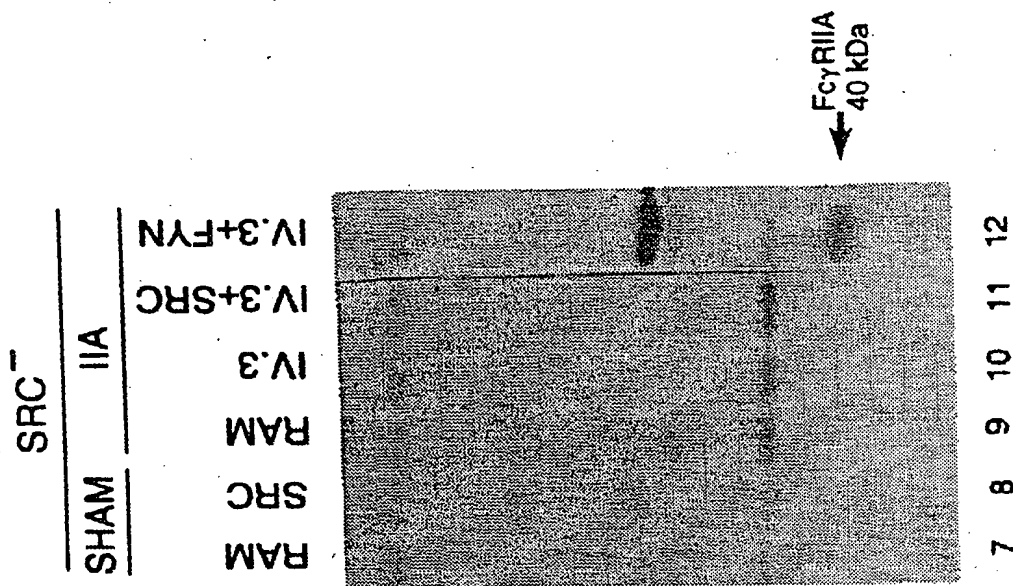
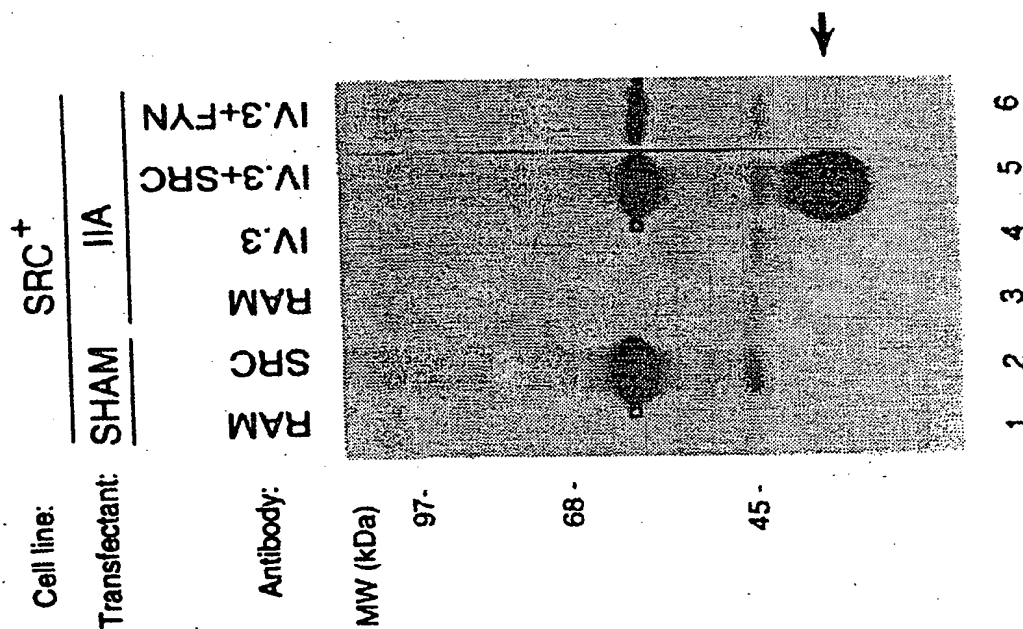
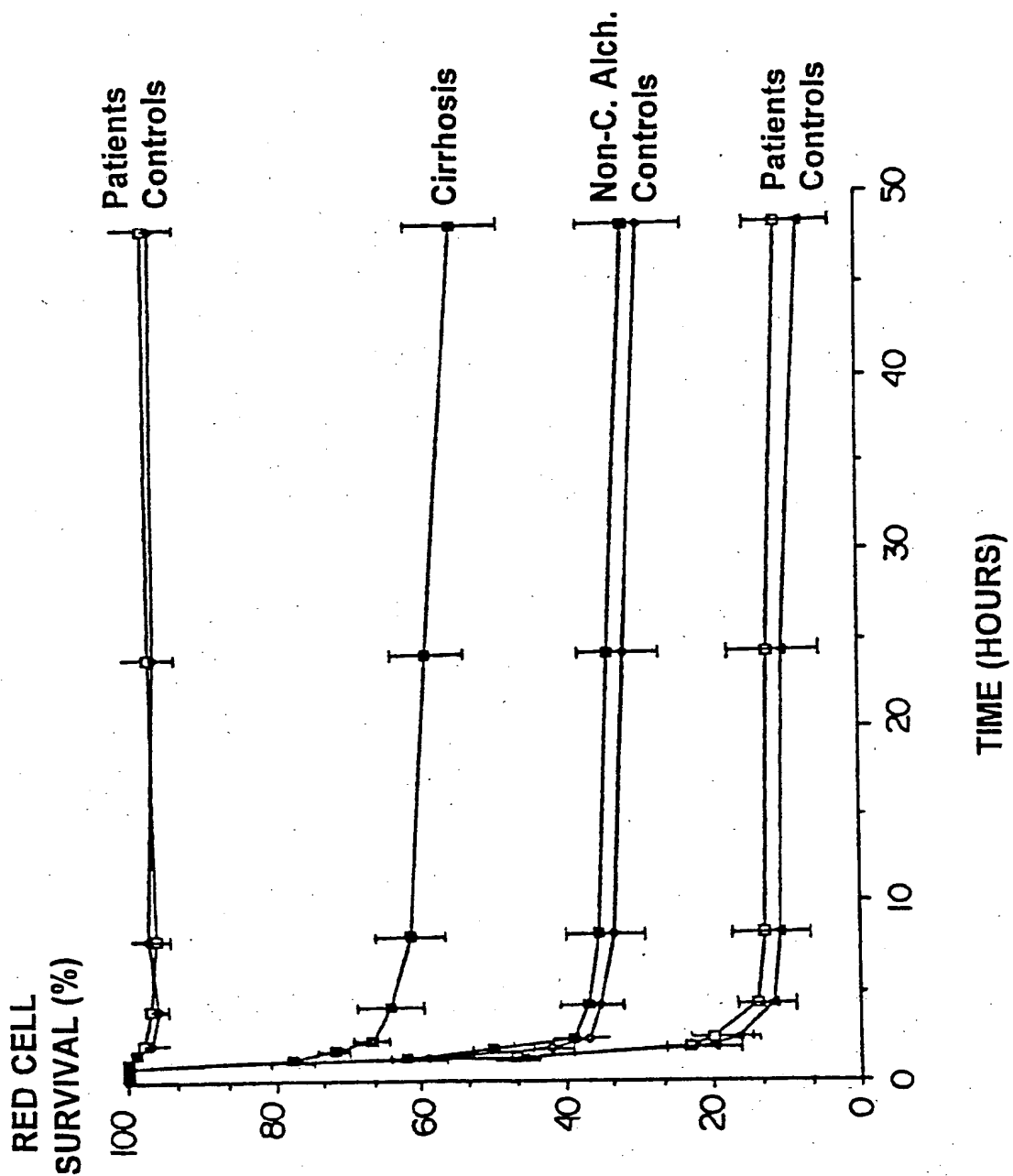


Fig. 3A



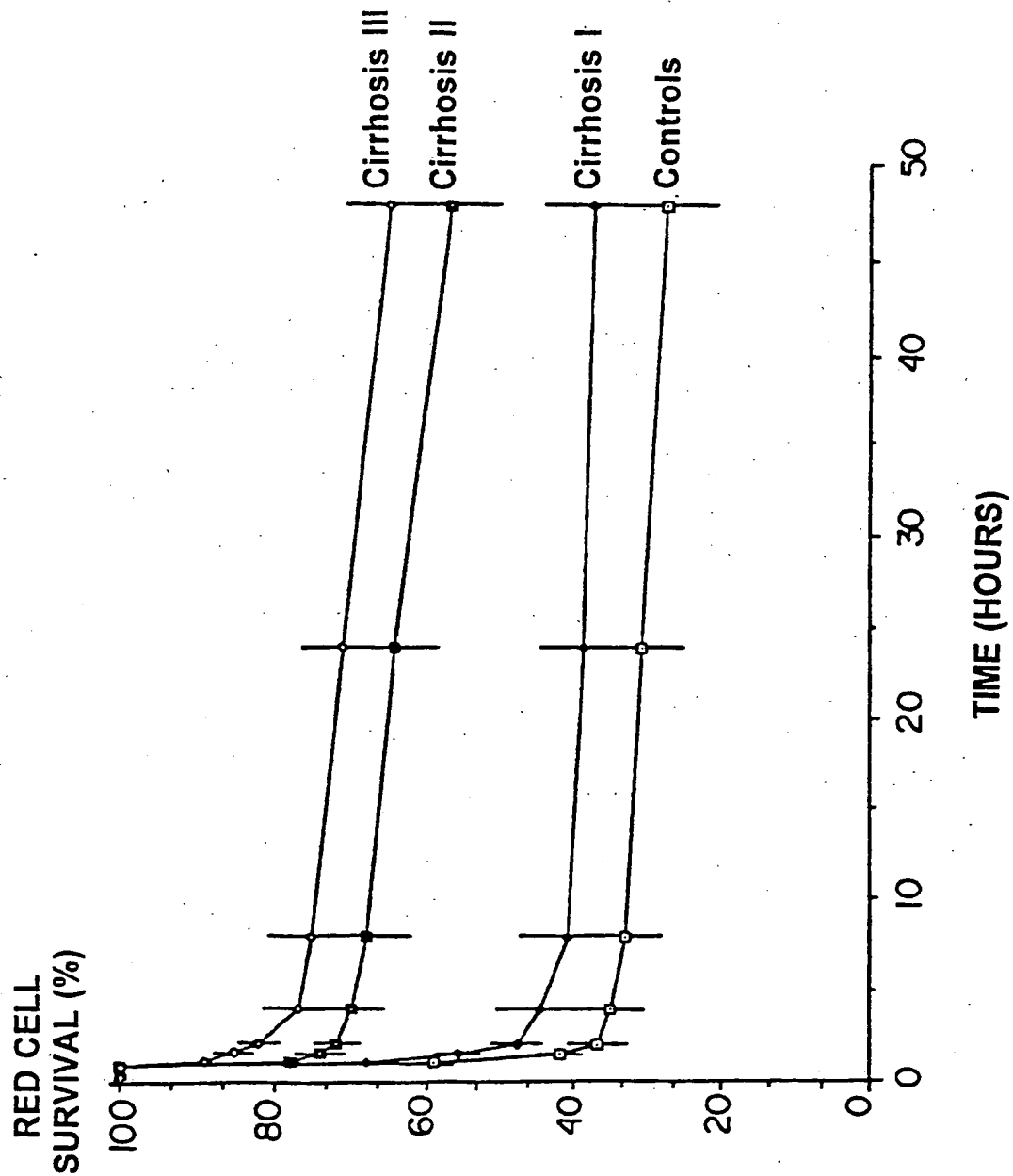
4 / 1 2

Fig. 4



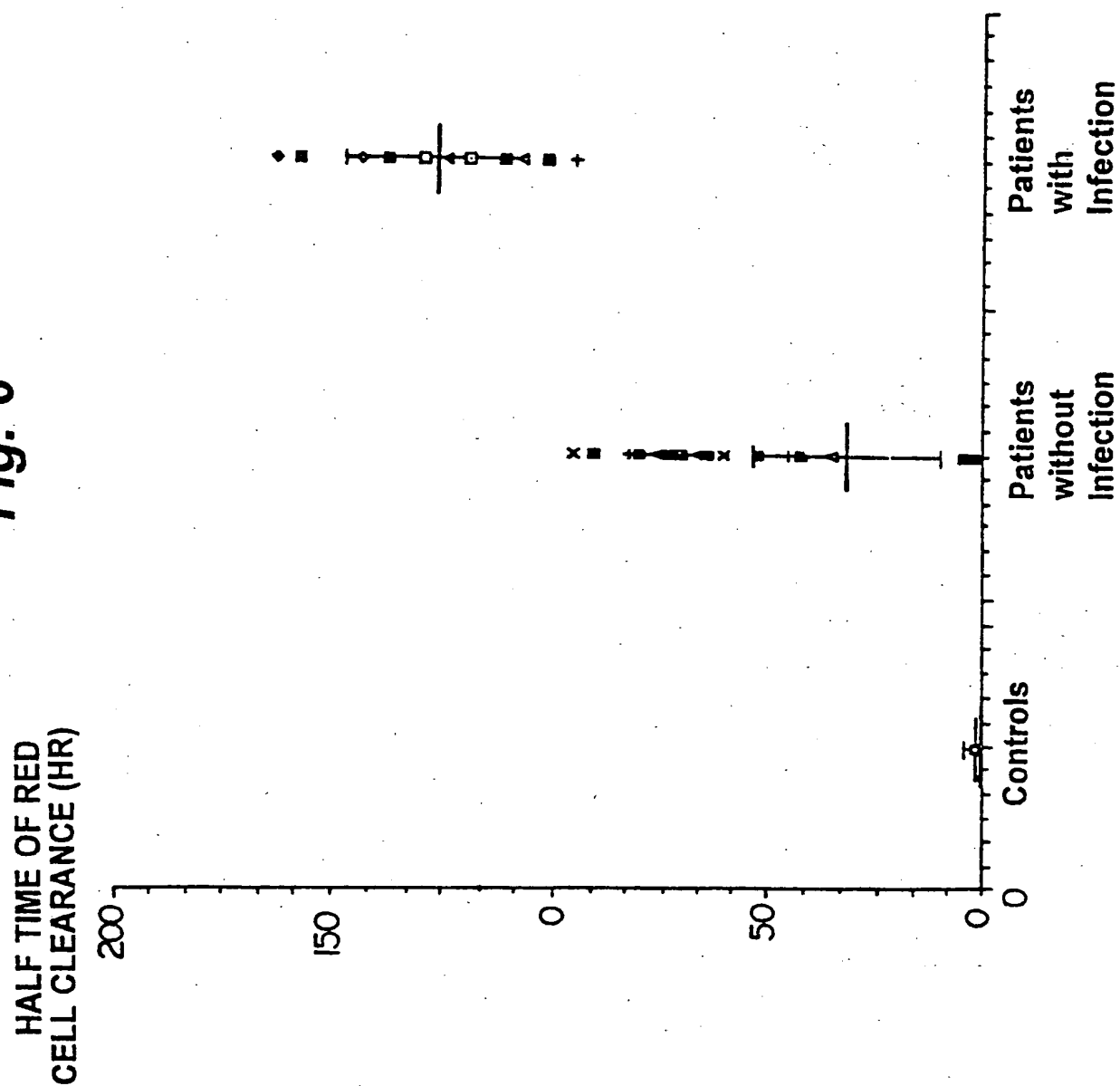
5 / 1 2

Fig. 5



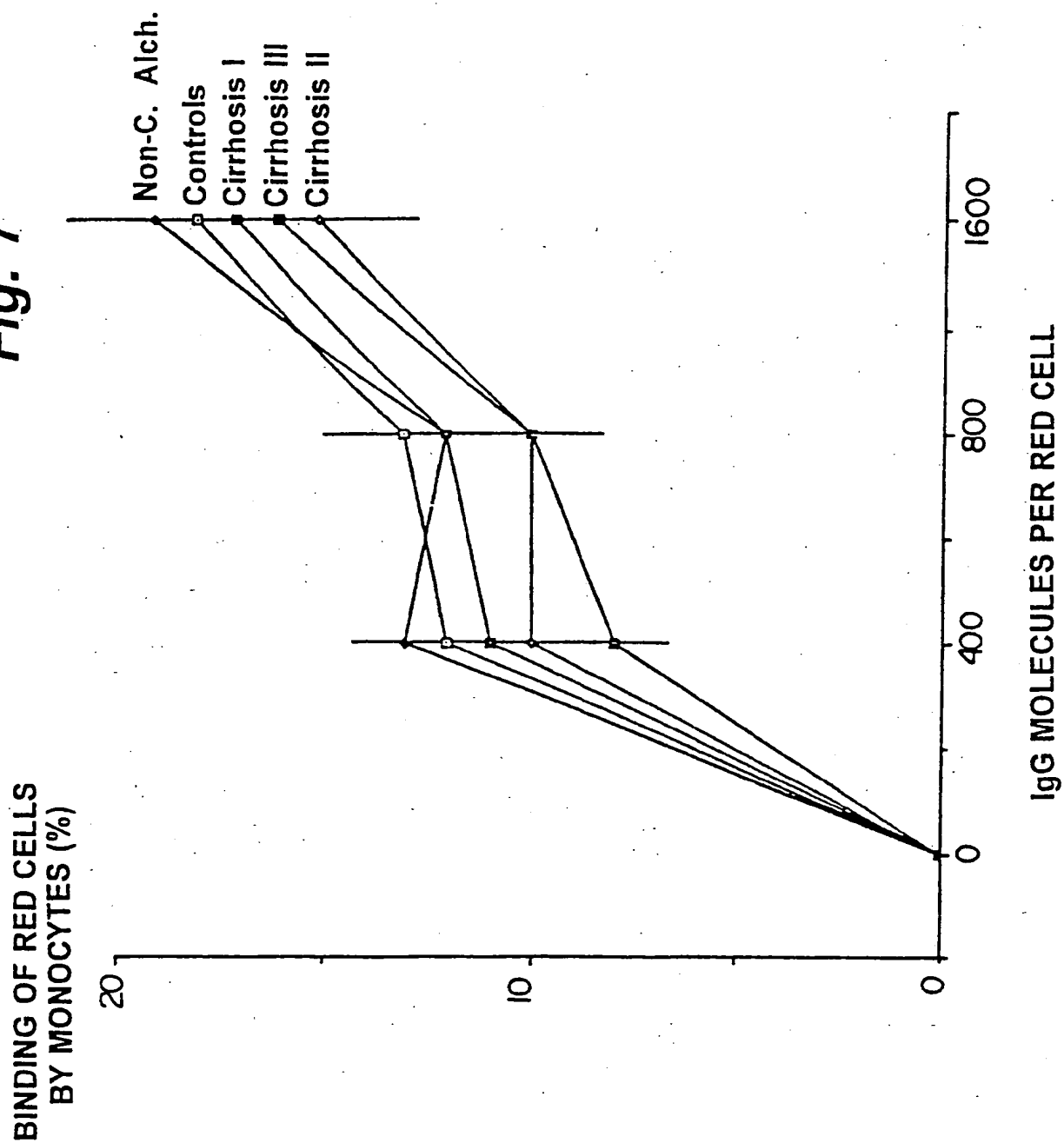
6 / 1 2

Fig. 6



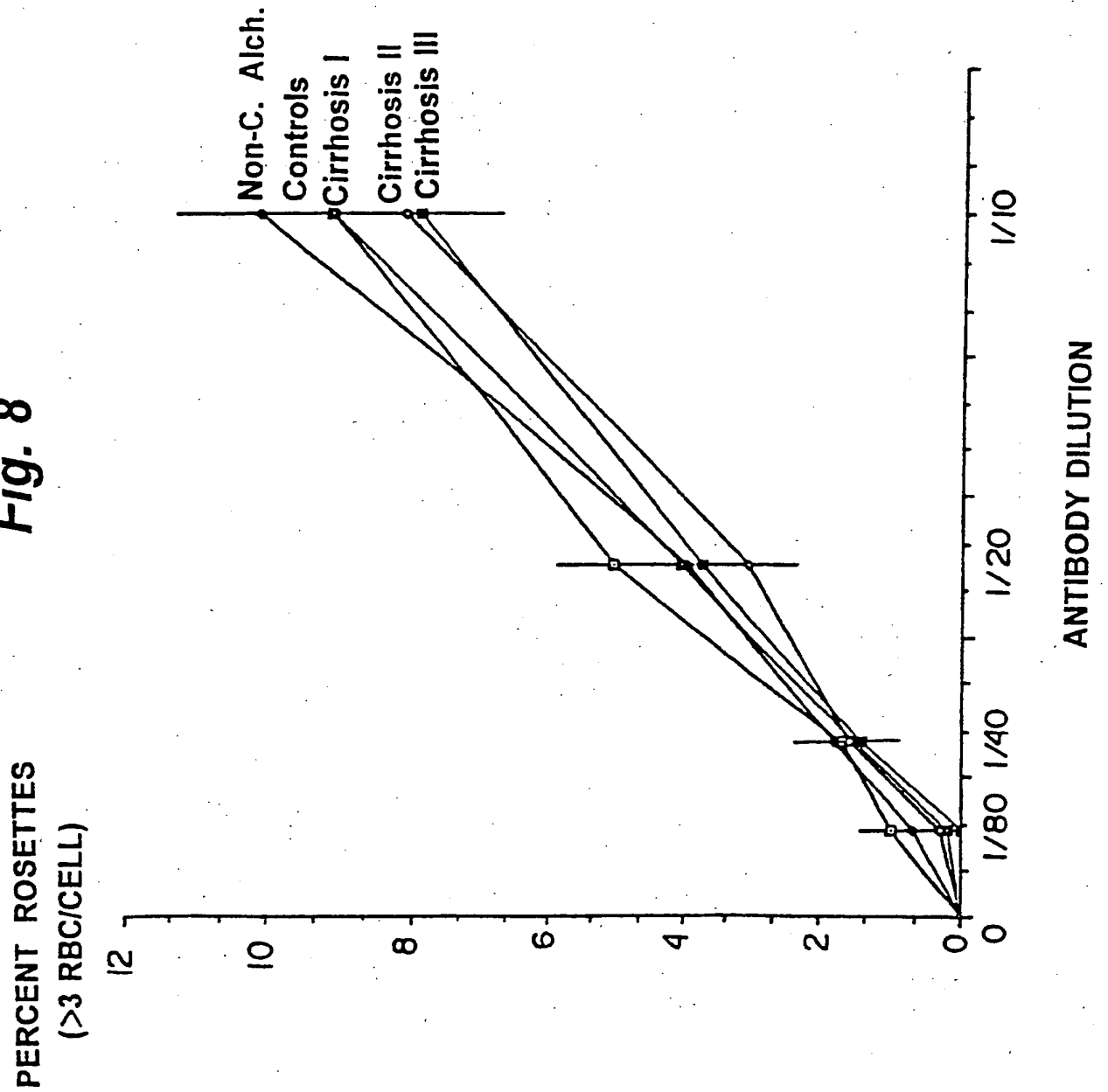
7 / 1 2

Fig. 7



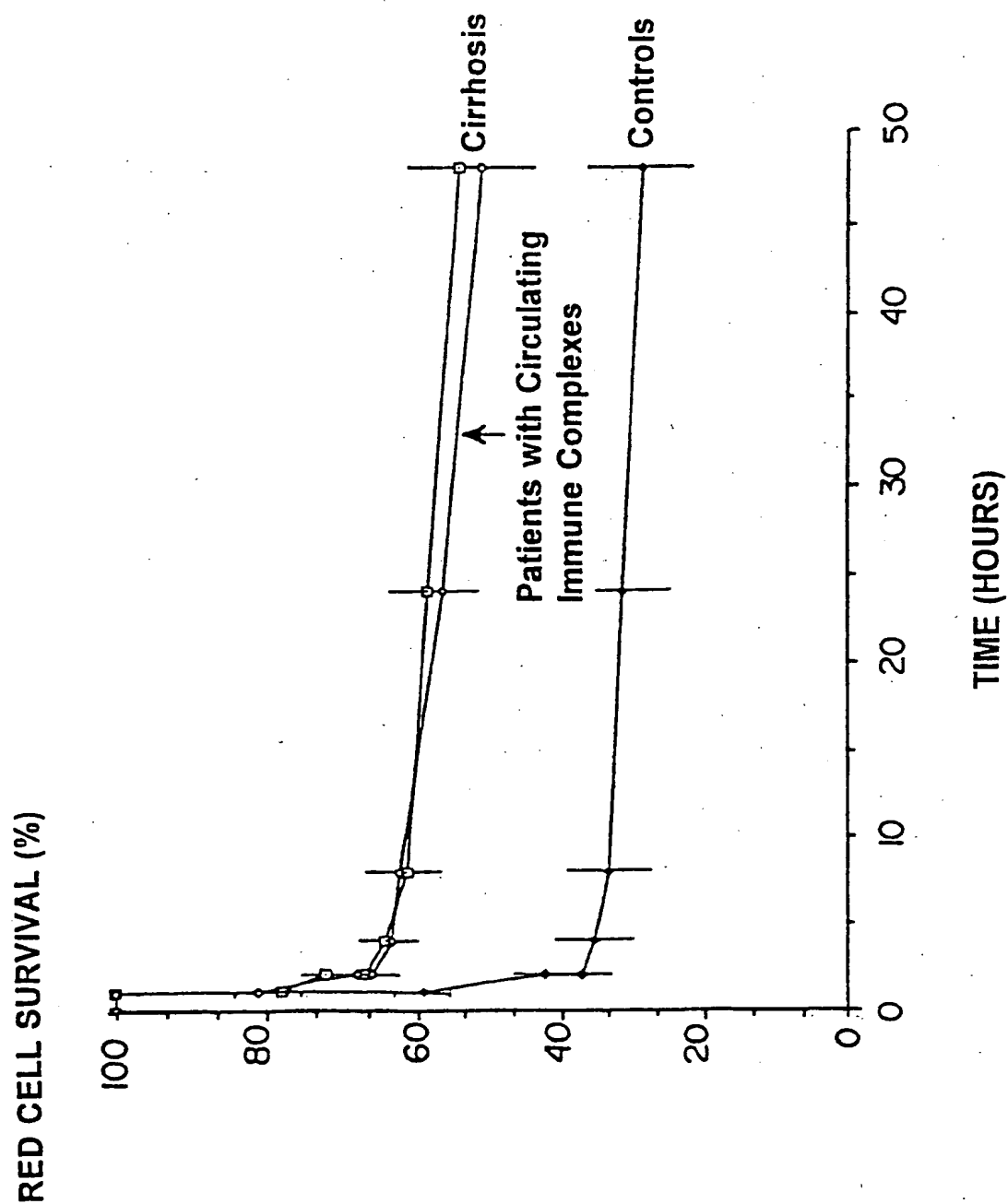
8 / 1 2

Fig. 8

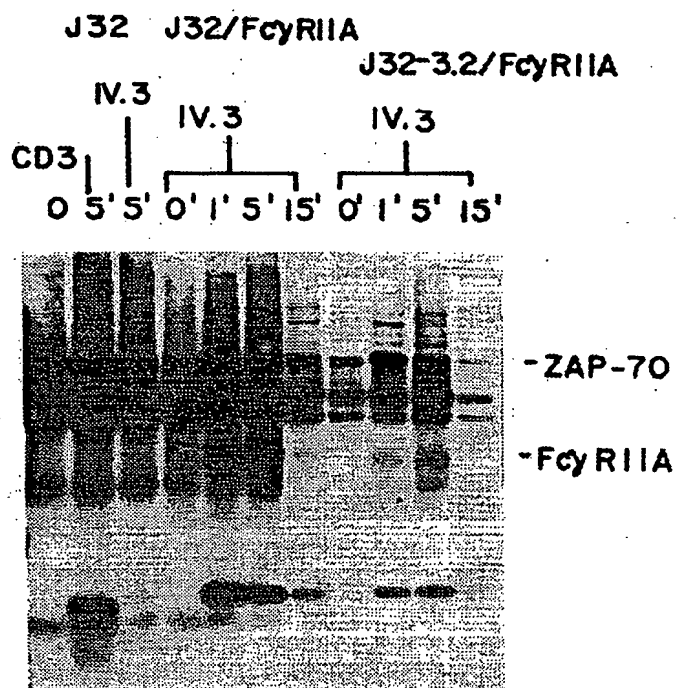


9 / 1 2

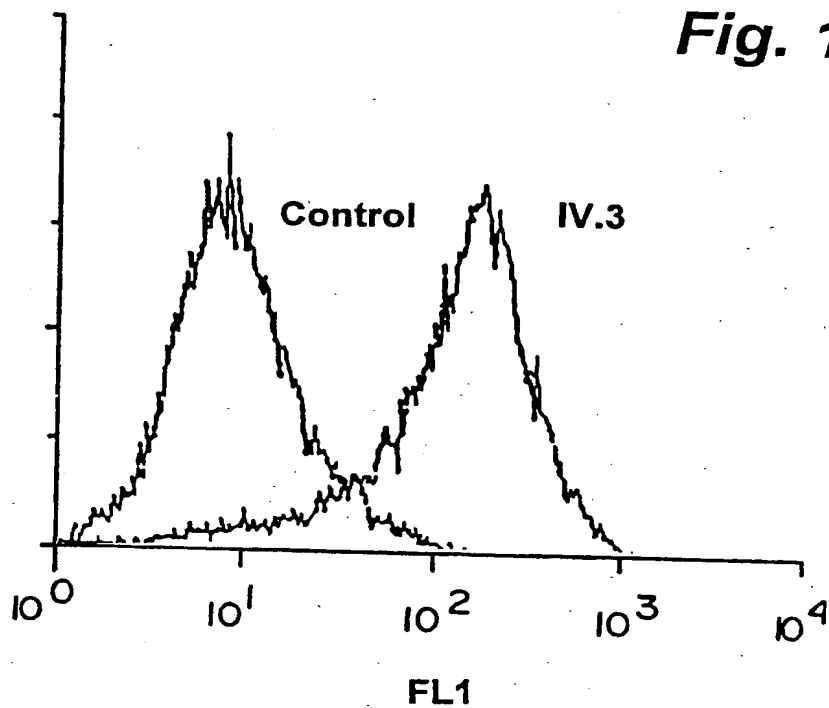
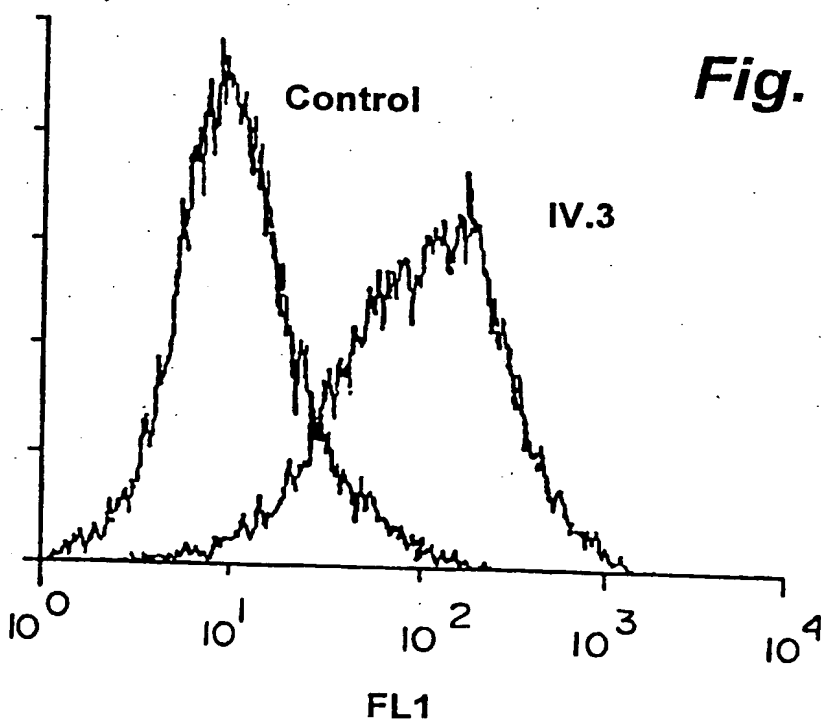
Fig. 9



10 / 12

Fig. 10

11 / 12

J32/Fc γ RIIA**Fig. 11A**J32 - 3.2/Fc γ RIIA**Fig. 11B**

1 2 / 1 2

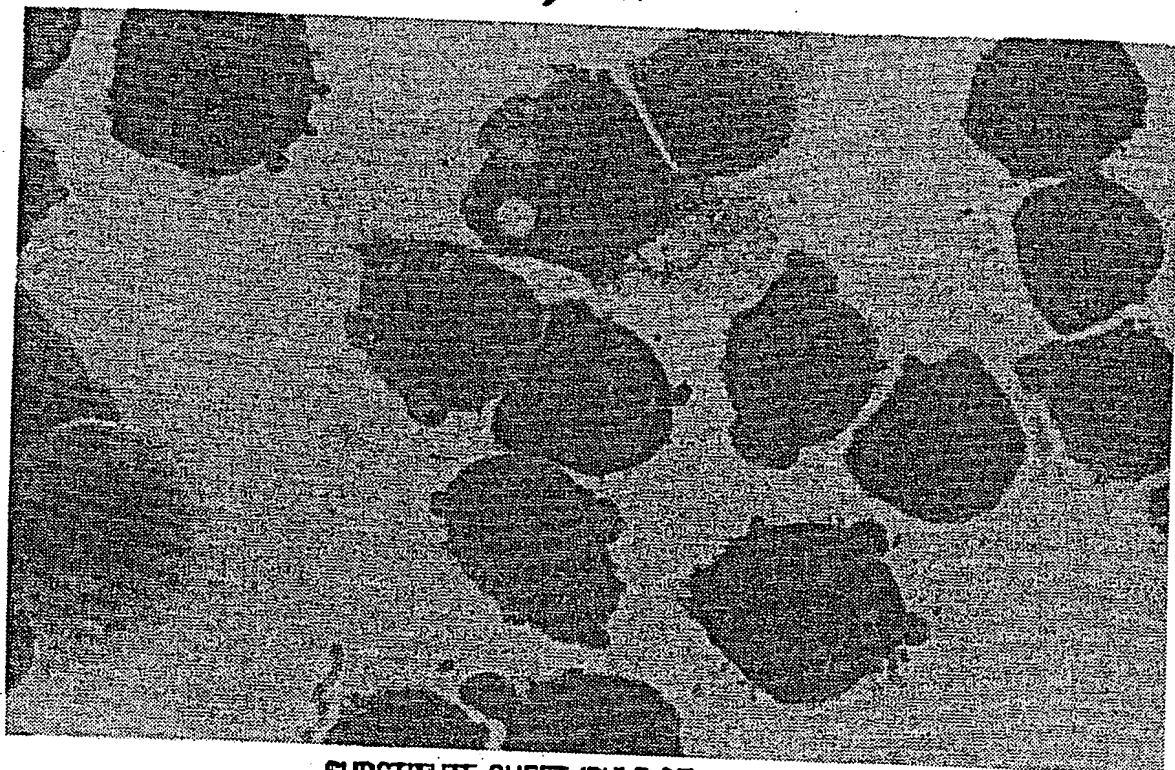
Fig. 12A

J32 / $\text{Fc}\gamma\text{RIIA}$



Fig. 12B

J32-3.2 / $\text{Fc}\gamma\text{RIIA}$



INTERNATIONAL SEARCH REPORT

International application No. .

PCT/US94/11111

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 15/00; C07K 14/00; C07H 21/00

US CL :514/44; 435/172.3; 530/350; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/172.3; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: fc receptor, phagocytic potential, liposome, Y-X2-L, syk

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 244, issued 16 June 1989, Friedmann, "Progress toward human gene therapy", pages 1275-1280, see entire article.	10, 18
Y	Journal of Clinical Investigation, Volume 75, issued January 1985, Friedman et al., "Effect of estradiol and steroid analogues on the clearance of immunoglobulin G-coated erythrocytes", pages 162-167, see entire article.	1, 5, 6, 8, 12, 13, 37, 42 and 43
Y	Journal of Clinical Investigation, Volume 88, issued November 1991, Indik et al., "Human FcγRII, in the absence of other Fcγ receptors, mediates a phagocytic signal", pages 1766-1771, see entire article.	1, 5, 6, 8, 12-17, 20, 21, 37, 42 and 43



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

10 JANUARY 1995

Date of mailing of the international search report

JAN 25 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SUZANNE ZISKA, PH.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11111

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 226, issued 26 October 1984, Anderson, "Prospects for human gene therapy", pages 401-409, see entire article.	9, 11, 19, 38 and 39
Y	GB, A, 2,223,755 (EVANS) 18 April 1990, see entire document.	9, 11, 19, 38 and 39
Y	Nature, Volume 358, issued 23 July 1992, Amigorena et al., "Tyrosine-containing motif that transduces cell activation signals also determines internalization and antigen presentation via type III receptors for IgG", pages 337-341, see entire article.	7, 40 and 41
Y	Cancer Research, Volume 51, issued 15 February 1991, Munn et al., "Role of low-affinity Fc receptors in antibody-dependent tumor cell phagocytosis by human monocyte-derived macrophages", pages 1117-1123, see entire article.	2-4, 22-26, 27-31, 32 and 33-36
Y, P	Biochemical and Biophysical Research Communications, Volume 200, No. 1, issued 15 April 1994, Yagi et al., "Cloning of the cDNA for the deleted SYK kinase homologous to ZAP-70 from human basophilic leukemia cell line (KU812)", pages 29-34, see entire article.	44-46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11111

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- Group I. Claims 1-13, 18, 19, 22-26, 32 and 37-43, drawn to a method of increasing the phagocytic potential of cells present in a mammal comprising introducing into said cells a DNA molecule coding for an Fc receptor, classified in Class 514, subclass 44, for example, and drawn to a DNA construct encoding an Fc receptor and a cell comprising the construct, classified in Class 536, subclass 23.1, for example.
- Group II. Claims 14-17, 20 and 21, drawn to a method of increasing the phagocytic potential of cells of a mammal, classified in Class 424, subclass 93.21, for example.
- Group III. Claims 27-31, 33-36, drawn to an Fc receptor comprising domains, classified in Class 530, subclass 350, for example.
- Group IV. Claims 44-46, drawn to a method of increasing the phagocytic potential of a cell comprising introducing into said cell a construct comprising a nucleotide sequence encoding the Syk gene, classified in Class 514, subclass 44 or Class 435, subclass 172.3, for example.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Invention II is drawn to the administration of cells to a mammal and involves different materials and procedures than does the method of Invention I or Invention IV. Invention III, drawn to the Fc receptor protein is a product not required for the practice of any of Inventions I, II or IV. Each grouping of claims forms a separate invention not linked to form a single inventive concept. PCT Rule 13 does not permit multiple distinct products and methods within a single application.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

This Page Blank (uspto)